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# **ROLE OF THY-1 GLYCOPROTEIN IN NEURITE OUTGROWTH**

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Thesis submitted in partial fulfilment of the requirements  
of the Open University for the degree of Master of Philosophy

December 1997

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*Om Prakrutimataye Namaha*



## ABSTRACT

In the peripheral nervous system (PNS), neurons regenerate axons following injury and reinnervate their target tissue. The substrate along which these axons extend is of critical importance. Mammalian central nervous system (CNS) neurons, normally unable to regenerate, can do so if given a permissive substrate. Adult axons will regenerate on embryonic CNS tissue and embryonic axons can grow in adult CNS environment but generally adult axons do not regenerate on adult glia. There are inhibitory molecules in the mature CNS environment that regulate growth. In this study I have examined the mature astrocyte-adult axon paradigm.

Thy-1, a glycoprotein consisting of 110 amino acids is present on mature axons. When transfected into neuroblastoma cell line NG115/401L, it inhibits neurite outgrowth on a monolayer of mature astrocytes. I have used primary sensory neurons derived from Thy-1 knockout mice to study the inhibitory effect of Thy-1. I have modified the original method for dissociating dorsal root ganglion cells from adult mice resulting in a population of DRG neurons that grow on average six times longer at 24 hours than other reported measurements. Using an image analysis system for measuring total neurite outgrowth, I have shown that Thy-1 does not prevent but significantly restricts neurite outgrowth over astrocytes. Preliminary experiments on embryonic DRG cells suggests that at a stage in development when Thy-1 is not present on neurons there is no difference in neurite outgrowth between Thy-1 positive and Thy-1 negative DRG cells. A growth restrictive role for Thy-1 is thus indicated. To fully understand the role of the axon-astrocyte interplay I have developed a three-dimensional *in vitro* system to study the extent to which Thy-1 is involved in this system.

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## **ABBREVIATIONS**

AraC	Cytosine Arabinocide
BSA	Bovine serum albumin
CIB	CO <sub>2</sub> - independent medium + 0.5% BSA
COS	Monkey Kidney Fibroblast cell line
DAPI	4', 6-diamidino-2-phenyl indole
EDTA	Ethylene diaminetetracetic acid
FCS	Foetal calf serum
FITC-SAR	Sheep anti rabbit IgG conjugated to fluorescein isothiocyanate
FITC-RAR	Rabbit anti rat antibody conjugated to fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
GAP 43	Growth associated protein
IAA	Isoamyl alcohol
IPA	Iso propyl alcohol
MAG	Myelin-Associated Glycoprotein
MEM	Minimal Essential Medium
NGF	Nerve Growth Factor
PBS-CM	Calcium and Magnesium free Phosphate buffered saline
SDS	Sodium dodocylsulphate
S-MEM	Spinners Minimum Essential Medium
S-MEMF	Spinners Minimum Essential Medium + 10%FCS
TRITC-RAM	Rabbit anti mouse antibody conjugated to tetramethylrhodamine isothiocyanate


# **CHAPTER 1**

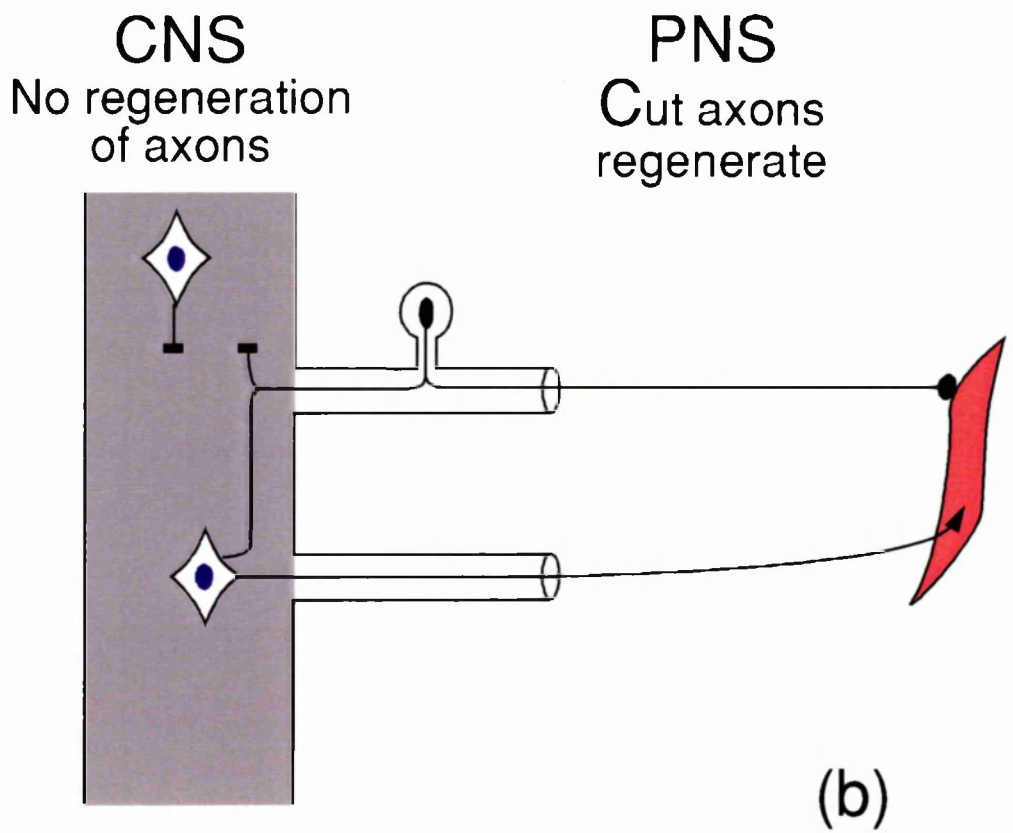
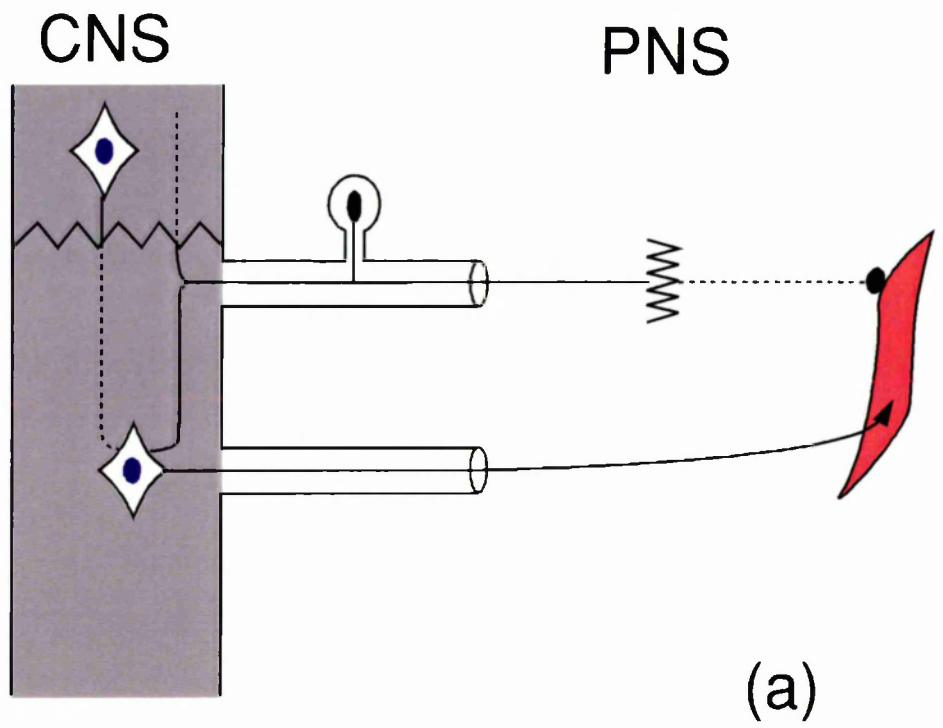
## **INTRODUCTION**

### **I REGENERATION-THE PROBLEM**

Spinal injury and blood clot in the brain are examples of injury of the central nervous system (CNS). Here there is damage to nerve connections and function can be irreversibly disturbed. Damage to the peripheral nervous system (PNS), for example a severed finger can be surgically repaired and here damaged nerves reconnect.

Axons if lesioned or damaged in the CNS of adult vertebrates fail to regenerate; if lesioned in the peripheral nervous system they can regenerate well (Cajal, 1928) and in many cases eventually restore functional connections (Vidal-Sanz *et al.*, 1987). The most striking example of dichotomy in regenerative capacity of PNS and CNS is illustrated by the sensory neurons of the dorsal root ganglia (DRG). The cell body resides in the periphery, while the axon bifurcates, one branch going to the peripheral sense organs, the other entering the CNS environment of the spinal cord at the dorsal root (Fig 1). If this centrally directed axon is cut in the periphery, it will regenerate up to, but not enter, the astrocytic environment of the CNS (Liuzzi *et al.*, 1987; Carlstedt *et al.*, 1989). If a central branch is cut within the CNS regeneration does not occur. If a peripheral branch is cut it has the capacity to regenerate and connect to target. Uninjured axons within the spinal cord grey matter have limited ability to sprout into territory vacated by lesioned dorsal roots (McMahon, 1992).

**FIG 1.** Regeneration in the peripheral and central nervous system. Axons from the dorsal root ganglion (DRG) bifurcate into two branches a short distance from the cell body. The peripheral branch is the sensory process that leads from the muscle spindle and the central branch extends through the dorsal root to the spinal cord. If the peripheral branch is cut () in the PNS (a), it regenerates to form functional connections (b). If the central branch is cut in the CNS (a) it does not regenerate (b).



To regenerate, neural cells have to survive; functionally appropriate neural pathways have to be re-established; severed axons must sprout, regrow across the lesion and then find and re-innervate their appropriate post-synaptic targets to form functional connections. A limited injury in grey matter may affect several thousand neurons, and because the process of neurogenesis is for most neurons a single phase that stops during embryonic or early postnatal life, the damaged neurons are not replaced and this may lead to some functional incapacity. The most devastating injuries in the CNS however, are usually not those to the neuronal cell bodies, but due to lesions in axonal tracts in the white matter. Here, even small injuries can sever millions of axons. Often particular functions will be totally lost, due to the inability of lesioned CNS axons to regenerate.

In the developing and young adult CNS, the glial environment is involved in channelling axons through the correct pathways in the fibre tracts and, in the terminal fields, glial cells (eg., astrocytes) interact with axons to stabilise and maintain nerve connections and eventually form synapses. In the mature CNS however, the same glial environment restricts regeneration following axonal injury. The problem of repair of damaged axons in the CNS is one of the mature vertebrate system where there is lack of growth promoting factors and/or abundance of growth inhibitory factors. Thy-1, which is expressed on axons after they have finished growing (Xue *et al.*, 1990, 1991) may be involved in restricting axonal growth and contributing to stabilisation of synaptic connections in the mature CNS environment.

For successful regeneration in adult, a growth permissive environment similar to that found in the PNS and the developing CNS has to be recreated. Long-range growth and guidance to target in mature CNS may continue to impose critical constraints on the recovery of useful connectivity in the injured mammalian CNS. We need to understand factors which affect survival of

damaged neurons, molecular differences between the adult peripheral and central environments and between adult and developing neurons. This understanding will allow development of intervention strategies that can promote axonal growth and thereby repair of the damaged nervous system.

## **II                    REGENERATION IN LOWER VERTEBRATES AND** **MAMMALS**

During development, differentiating neurons from the CNS and PNS send out axons to their targets either in the periphery or within the CNS. In amniotes, this stage of neurogenesis is completed during the embryonic phase of life, but in anamniotes, there is a continuous neuronal growth and addition of neurons to the established adult nervous system (Holder and Clarke, 1988). Regenerative ability of axons is retained in lower vertebrates and invertebrates.

Retinal fibers of cut optic nerve of a frog grow back to optic tectum and form appropriate synapses and vision is eventually restored (Attardi and Sperry, 1963). When toad retinal ganglion neurons are axotomised they regenerate, axons regrow accompanied by molecular changes. Skene observed an increase in specific activity of radiolabelled GAP-43 which is a growth associated protein related to axon growth (Skene and Willard, 1981). Similarly, after interruption of the optic nerve in the goldfish, vision is restored as axotomised retinal ganglion cells regenerate and synapse appropriately with nerve cells in the optic tectum (Grafstein, 1986). Further in the goldfish, spinal cord transection does not lead to the formation of a dense glial scar as in adult mammal. Here, nerve fibres regenerate successfully across the lesion and the goldfish returns to normal swimming in 20-40 days (Coggeshall and Youngblood, 1983). Glial cells of the goldfish optic nerve

express high levels of laminin and L1-like cell adhesion molecule and secrete factors that may contribute to stimulating and promoting regeneration, and little or none of inhibitory molecule that is present in the CNS environment in mammals (Bastmeyer *et al.*, 1993; Wanner *et al.*, 1996; Schwab *et al.*, 1996).

Complexity and specialisation endowed by evolution means that regeneration and functional connection of cut CNS axons in higher adult vertebrates is nearly always impossible. The lack of regenerative capacity of adult amniote CNS is due to special characteristics of both neurons and glia. The cutting of the optic nerve in mammals results in retrograde degeneration of many retinal neurons (Richardson *et al.*, 1982). The retinal axons here do not regrow and these and other injured neurons in the adult mammalian brain and spinal cord remain permanently disconnected from their natural fields of innervation.

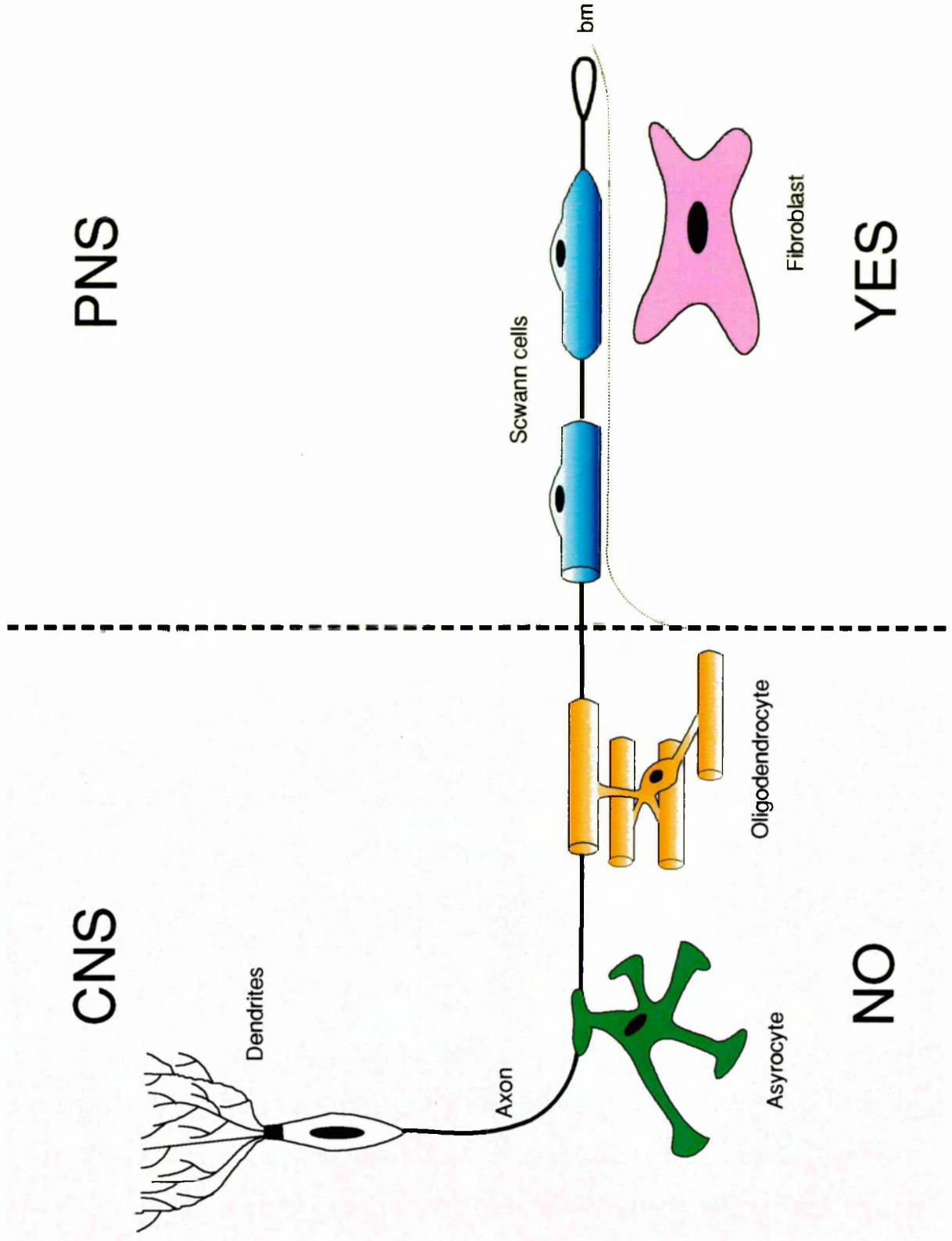
In higher vertebrates early in development there is overproduction of neurons. Further growth is down-regulated and cells die shortly after they start innervating target fields when a genetic programme for cell death is activated. This process matches the number of neurons to the requirements of the target fields and eliminates inappropriately connected neurons (Cowan *et al.*, 1984). Later in development, post-natal experience driven selection is used to retain those connections that are most appropriate and these alone survive and are maintained in the adult (Purves, 1988). Aberrant growth is prevented by inhibitory molecules (Schwab *et al.*, 1988). There is stabilisation of useful connections in the neuron-astrocyte environment. This selection of appropriate neurons and connections during development is restricted to higher vertebrates. In lower vertebrates there is a precise number of neurons matched to exactly the same number of target cells and pruning of unmatched neurons is unnecessary. This system has no need for the inhibitory mechanism required to control over-exuberant growth and this may be why there is axonal growth in lower vertebrate CNS.

On observing the failure of DRG axons to regenerate into the CNS, Ramón y Cajal concluded that, 'The tendency to restoration (of lesioned nerve fibres) is frustrated by two negative conditions: (1) the lack of substances able to sustain and invigorate the indolent and scanty growth of the sprouts; (2) the absence in the path of interrupted nerve fibres of catalytic agents capable of attracting and directing the axonic current to its destination (Cajal, 1928). Cajal's conclusions were experimentally tested by Aguayo. His group transplanted a segment of peripheral nerve to a central site and vice versa. They showed that in the presence of PNS graft, central axons can actually extend many millimetres after an injury but are prevented from doing so by some aspect of the central environment. When central axons confronted with a distal stump of a peripheral nerve they regenerate almost as well as severed peripheral axons (Bray *et al.*, 1981; Benfey and Aguayo, 1982). Many different types of CNS neurons can extend a long distance if presented with the right environment. Bridges of peripheral nerve can serve as bypass conduits to route at least some axons across spinal lesions in rats (David and Aguayo, 1981). Adult rat retinal ganglion cell neurons reinnervate peripheral nerve segments grafted into the rat brain (Richardson, McGuinness and Aguayo, 1980). Subsequent reconnection to postsynaptic targets was shown in the optic system (Kierstead *et al.*, 1989). These experiments showed that the severance of axons in the higher vertebrate does not always result in neuronal death and under particular conditions neurons do regenerate their axonal projections and re-establish contact with proper targets in brain and spinal cord.

So why do cut axons regenerate in the PNS environment but not in the CNS? The PNS environment is dominated by a glial cell type known as the Schwann cells (Fig 2) and the CNS environment consists of astrocytes and oligodendrocytes.



**FIG 2.** CNS-PNS environment. The immediate environment encountered by growth cones is of prime importance in axonal regeneration. Axon encounters oligodendrocytes and astrocytes in the CNS and Schwann cells and fibroblasts in the PNS. Schwann cells provide a growth permissive environment in the PNS and here there is regeneration of damaged axons. In the CNS, the oligodendrocytes and the astrocytes restrict axonal growth; here, cut axons do not regenerate.



Schwann cells may be responsible for promoting growth from neighbouring neurons both in the peripheral graft experiments (Richardson *et al.*, 1980) and when cultured Schwann cells are implanted into various sites in the CNS (Kromer *et al.*, 1985; Xu *et al.*, 1994). Considerable axotrophic effect of adult Schwann cells has also been observed after transplantation into lesioned adult rat thalamus (Brook *et al.*, 1994). Regenerating axons follow processes extended by Schwann cells in response to denervation and they play a primary role in initiating sprouting and in guiding the growth of the sprouts after denervation (Son and Thompson, 1995).

The inhibitory role of the cellular components of the CNS was demonstrated in co-culture experiments using dissociated glial cells and a variety of cultured neurons. Growth inhibition here was dependent on physical contact with oligodendrocytes and was not due to soluble factors in oligodendrocyte conditioned medium (Caroni and Schwab., 1988). Reactive astrocytes are one of the main components of scars which are formed as a result of traumatic CNS lesion (Reier *et al.*, 1983). Regenerating fibers at lesion sites and in areas of tissue transplants show that dense astrocytic scars form a physical barrier and inhibit neurite growth (Jakeman and Reier, 1991). Local sprouting of lesioned adult rat spinal cord axons has been shown to persist in the presence of astrocytic scarring and this may be due to massive expansion of cut end of lesion presenting a large area for expression of receptors for growth promoting molecules (Li and Raisman, 1995).

As Schwann cells from the peripheral nerve produce a variety of neurotrophic factors and even increase this production after denervation (Richardson *et al.*, 1982a; Heumann *et al.*, 1987), it was postulated that a difference in trophic factor production between the PNS and CNS is yet another factor that controls regeneration. However, identified neurotrophic factors like NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), CNTF (ciliary neurotrophic factor), and FGF (fibroblast growth

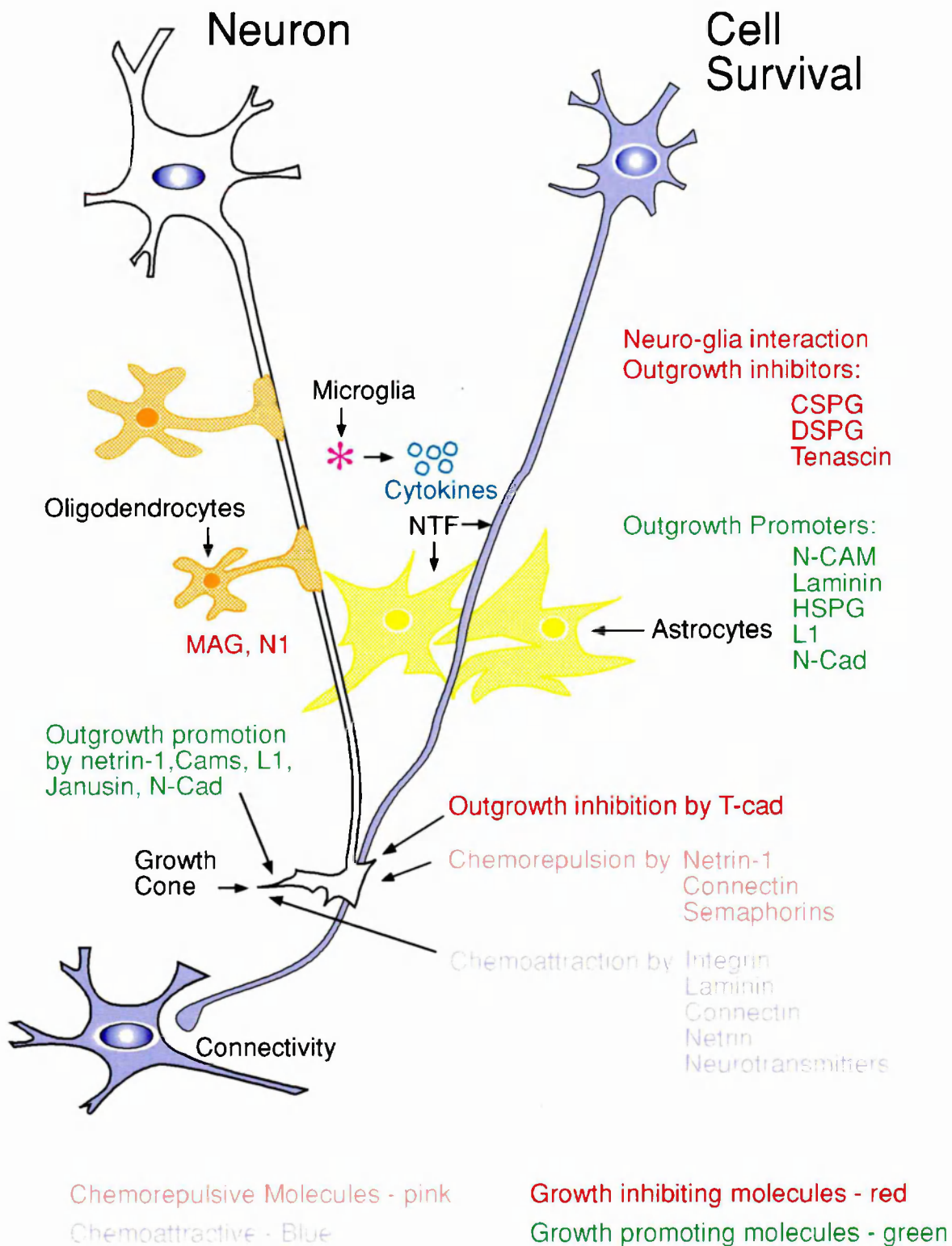
factor) were found to be present in the adult CNS (Barde, 1989; Leibrock *et al.*, 1989; Stöckli *et al.*, 1989), and increases were also found at central lesion sites (Needels *et al.*, 1986; Whittemore *et al.*, 1987). In elegant but simple experiments it was demonstrated that the presence or absence of neurotrophins however, may not be the only factor in controlling regeneration. When dissociated perinatal rat sensory or retinal sympathetic neuron were co-cultured with explants of adult optic or sciatic nerve in the presence of neurotrophic factors, no axons were found in the optic nerve but axon ingrowth into the peripheral nerve was abundant (Schwab and Thoenen, 1985). Trophic factors which can potentially promote growth are present in the CNS and the PNS but there is present in the CNS environment an excess of growth inhibitory factors and the effect of inhibitory factors in the adult CNS environment can not be overcome by stimulatory effects of neurotrophic factors.

Neuron survival, growth and connectivity are essential for regeneration and involves a complex series of cellular and molecular interactions (Fig 3).

The basal lamina of Schwann cells contain laminin and fibronectin which are potent promoters of neurite growth in culture (Bozyczko and Horwitz, 1986). This basal lamina containing cell surface molecules L1/Ng CAM (neuron-glia cell adhesion molecule), N-Cadherins and integrins, the major immunoglobulin superfamily of transmembrane proteins have shown to be involved guidance and growth promoting property of the PNS (Seilheimer and Schachner, 1988; Bixby and Harris, 1991; Doherty and Walsh, 1992). Antibodies to the cell surface molecules when applied together masked the neurite promoting ability of Schwann cells (Seilheimer *et al.*, 1988). Although neural cell adhesion molecule L1 mediates neurite outgrowth via homophilic interactions (Williams *et al.*, 1992), the heterophilic binding of neuronal L1 to substrate-associated TAG-1 (Felsenfeld *et al.*, 1994) also stimulates neurite

**FIG 3.** Schematic diagram of molecular and cellular interactions involved in regeneration of the adult mammalian CNS. Cell survival, axonal elongation, chemorepulsion, chemoattraction and connectivity are events that occur during regeneration. A growing axon encounters astrocytes (yellow), microglia (purple) and oligodendrocytes (orange). Molecules denoted in green print are outgrowth-promoting (N-CAM, Neural cell adhesion molecule; Laminin; HSPG, heparan sulphate proteoglycan; L1; N-Cad, neural cadherin; Janusin) and in red print are outgrowth-inhibiting molecules (CSPG, chondroitin sulphate proteoglycan; DSPG, Dermatan sulphate proteoglycan; Tenascin; T-Cad, truncated cadherin). Molecules printed in pink and purple have chemoattractive (laminin; integrin; connectin; netrin; neurotransmitters) and chemorepulsive (Netrin-1; connectin; semaphorin) properties respectively.

Adopted from review by I. Aubert., J.L. Ridet and F.H. Gage. *Current Opinions Neurobiology* (1995). Vol 5, page 625-635.



outgrowth. Integrins serve as receptors for a variety of extracellular matrix and cell surface molecules and they appear to be particularly important for neural development and several of their ligands (e.g. laminin, fibronectin and collagen) have been implicated in proliferation and migration (Galileo *et al.*, 1992) of neurons and the outgrowth of axons (Sanes, 1989). Role of Schwann cells' growth promoting molecules in regeneration was further implicated when experiments showed that antibody to laminin-heparan sulphate proteoglycan complex inhibit the growth of neurites on sections of peripheral nerve (Sandrock and Matthew, 1987). Further evidence for the importance of laminin in regeneration came from *in vitro* studies where neurite promoting ability was inhibited or precipitated from Schwann cell conditioned media by antibodies to laminin (Lander *et al.*, 1985).

In the adult CNS, growth restricting activity is associated with CNS myelin derived from oligodendrocytes and glial scar of astrocytic origin. In experiments where regenerating retinal ganglion cell axons are guided to the cerebellum of adult hamsters it was observed that the regenerating RGC axons grew predominantly in the cerebellar cortex, while extension into the white matter was exceptional and limited (Zwimpfer *et al.*, 1992). This observation is consistent with other studies suggesting that myelin component of the CNS of adult mammals inhibit cell adhesion and neurite outgrowth *in vitro* (Caroni and Schwab, 1988) and may block neurite extension *in vivo* (Schnell and Schwab, 1990). Neutralisation of growth inhibitory properties of CNS myelin by IN-1 antibody has shown partial functional recovery in adult rats of at least some motor function after injury to the spinal cord (Bergman *et al.*, 1995). This group reported that the recovery was associated with regeneration of brain stem spinal and corticospinal axons. This study however, did not present any evidence to suggest that the cut corticospinal fibres induced to regenerate actually establish synaptic connections and are responsible for functional connections. It is possible that collateral outgrowth of new axons from uninjured neurons may have contributed to recovery after a partial spinal injury. In the presence of neurotrophic factor NT-3 and neurite growth inhibitory activity

blocking antibodies, Schwab's group (Schnell *et al.*, 1994) reported enhanced sprouting of the corticospinal tract axon and 5-10 % axonal elongation in young adult rats. As well as NI-35/250, J1-160-180, cytactin, tenascin, MAG (Myelin Associated Glycoprotein) and chondroitin sulphate proteoglycans (CSPG) are some of the expanding group of candidate molecules which have been shown to be inhibitory in the CNS environment (Caroni and Schwab., 1988; Taylor *et al.*, 1993; Lochter *et al.*, 1991; Rudge and Silver, 1990; Pindzola, 1993; Brittis, 1992; Smith-Thomas, 1994; McKerracher *et al.*, 1994; Snow *et al.*, 1990). A collapsin/semaphorin gene family molecule that patterns sensory projections in chick spinal cord (Messersmith *et al.*, 1995) has been shown *in vitro* to cause growth cone collapse in chick DRGs (Luo and Raper, 1993). Its role in regeneration is not yet clear.

The mechanism of inhibition of neurite outgrowth has yet to be elucidated. As development progresses, growth cones are guided by several chemoattractive and chemorepulsive molecules expressed in a particular developmental time window (Kennedy, *et al.*, 1994; Colamarino and Tessier -Lavigne, 1995; Porter and Sanes, 1995). The net effect of cell-cell contact can be either permissive or inhibitory. In regeneration of adult mammalian axon in the CNS and in development, inhibitory molecules are able to prevent neurite attachment and outgrowth, or induce filopodial retraction and disruption of lamellipodial structure of the growth culminating in growth cone collapse or repulsion. Of the molecules known to cause axonal growth inhibition in the CNS, NI-35 has been shown with *in vitro* experiments to act by causing growth cone collapse (Bandtlow *et al.*, 1993). CSPG (Snow *et al.*, 1990) and J1-160/180 (Pesheva *et al.*, 1989) cause loss of adhesion to substrate either by inhibition of adhesive ligand-receptor interaction, or by interference with the surface charge interactions between the substrate and the growth cone that allow adhesion to take place (Johnson, 1993). Complex second-messenger systems involving G proteins and/or intracellular calcium ion concentration mediate ligand-receptor interaction involved with both forward growth and growth cone collapsing activity (Igarashi *et al.*, 1993; Brandtlow *et al.*, 1990).



During development, main fibre tracts (Schwab and Schnell, 1991) are formed with remarkable accuracy. Axon growth in the fibre tract is disciplined and intermingling of inappropriate fibres is prevented. Axon growth, collateral branching (Roskies and O'Leary, 1994) and synaptic connections in the terminal field during development are controlled by factors released from targets and by response to regulated expression of growth promoting and inhibitory cues and although short range one-to-one connections are made, a small but major degree of synaptic plasticity remains in adult brain to allow for adaptive responses such as learning (Pons *et al.*, 1991).

The axonal-glial interactions that regulate growth and stabilise connections in the developing brain prevent axonal regeneration in the adult CNS. The restriction of axonal regeneration depends upon the state of differentiation of the glial cells and neurons. This has been investigated *in vivo* by transplantation and *in vitro* by using tissue derived from mature and immature animals in a confrontation assay.

Axons regenerate more vigorously from explants taken from tissue during the period while axons are initially growing or from older explants whose axons have received a conditioning crush (Collins and Lee, 1982) and therefore have enhanced ability to regenerate due to availability in the axon of molecules associated with regeneration. Conversely, transplants fail in mature systems. Mature neurons do not regenerate axons along fibre tracts (Raisman *et al.*, 1985). The age-related failure of axons to grow could be due to developmental changes in the axons or in the glial environment.

Transplanted adult neurons die following transplantation into adult CNS, but axons have been shown to sprout in adult brain into which embryonic glial cells have been transplanted (Muller and Best, 1989). Partial innervation of foetal graft by host derived axons has also been observed (Raisman and Ebner, 1983; McLoon and Lund, 1983; Björklund *et al.*, 1987; Tessler *et al.*, 1988; Yasanobu and Tessler, 1990). Axons from embryonic neurons can, under particular conditions, grow into astrocyte-dominated grey matter of adult brain (Dunnett *et al.*, 1982; Zhou *et al.*, 1985; Björklund *et al.*, 1987) or the dorsal root entry zone in the spinal cord (Rosario *et al.*, 1993). Björklund observed that foetal or neonatal tissue from substantia nigra region of brain not only survived but differentiated, extended axons and released neurotransmitters after being inserted into cortex overlying the basal ganglia of the adult nervous system (Björklund and Stenevi, 1984). In experiments where foetal spinal cord was grafted into excised region of lower thoracic cord of 2 day old pups, (Iwashita *et al.*, 1994) extensive axonal connections and long lasting functional recovery were observed. Rosario's group showed that grafted embryonic dorsal root ganglia (DRG) neurons are capable of growing into adult spinal cord (Rosario, 1993). Human forebrain neuroblasts reform long axon pathways in lesioned adult CNS (Wictorin *et al.*, 1990) and long fibre growth is seen when embryonic mouse hippocampal neurons are transplanted into adult myelinated tracts (Davies *et al.*, 1993). Innervation of appropriate terminal fields is also seen when embryonic entorhinal transplants are placed in deafferented entorhinal zone of adult mouse hippocampus (Zhou *et al.*, 1989).

Response of embryonic and adult neurons to factors affecting neural growth may be different. Embryonic retinal ganglion cells extend processes on laminin but lose their responsiveness with maturation (Cohen *et al.*, 1986; Johnson *et al.*, 1988). Patterns of outgrowth of adult and embryonic DRG are different on the same peripheral nerve substrata (Bedi *et al.*, 1992). The difference in growth response between adult and embryonic neurons may be due to down-regulation of receptors (e.g. integrin) on adult retinal cells or perhaps embryonic neurons have not acquired receptors for factors which

inhibit the growth of their adult counterparts. Cohen (1991) reported that  $\alpha 6\beta 1$  integrin dimer mediated laminin-responsiveness of mouse RGC declines during embryonic development and is lost before birth. More recently it was demonstrated that rat neonatal dorsal root ganglion neurites failed but embryonic neurites grew over dorsal root entry zone substrata derived from P6 and adult rat (Golding *et al.*, 1996). It was observed that astrocyte and oligodendrocyte precursor cells as well as immature astrocytes were permissive but the late appearing oligodendrocytes and three-dimensional array of mature astrocytes were inhibitory for neurite growth (Schwab and Caroni, 1988; Fawcett *et al.*, 1989a and 1989b; Hatten *et al.*, 1990; Smith *et al.*, 1990). Further, Fawcett (1989a) showed that embryonic DRG penetrated and extended modest neurites in a three-dimensional culture of astrocytes but adult DRGs did not. Mature DRG are inhibited by myelin associated glycoprotein (MAG) but embryonic DRG grow well on cells transfected with MAG (Mukhopadhyay *et al.*, 1994). It appears that a combination of oligodendrocytes, mature astrocytes and adult axons is inhibitory for regeneration in the CNS.

## V

### **THY-1 STUDIES**

This *in vitro* study will focus on the role of Thy-1 in restricting axonal growth in mature astrocytic environment.

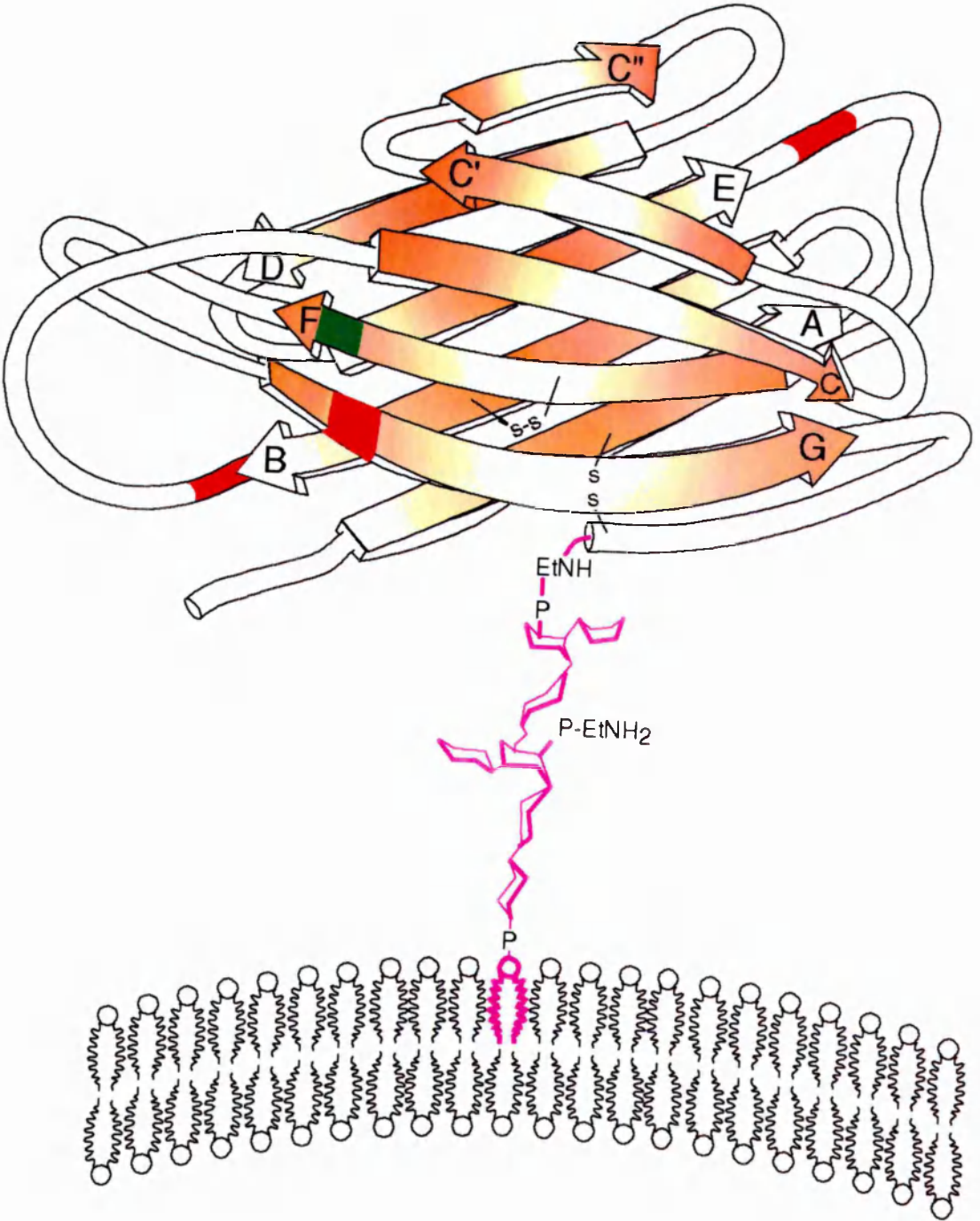
Thy-1 is the smallest known member of the immunoglobulin superfamily. In rats and mice, Thy-1 is the major cell-surface glycoprotein of thymocytes and a major glycoprotein of brain (Williams, 1989). The hypothesis that Thy-1 might play a role in contacts between neurons and their neighbours (Cohen *et al.*, 1981) was further strengthened by subsequent findings that other members of the immunoglobulin super

family such as N-CAM, L1 and MAG (Cunningham *et al.*, 1987; Moos *et al.*, 1988; Poltorak *et al.*, 1987) are involved in contacts between neurones and their neighbours (Faivre-Sarrailh and Rougon, 1997).

The Thy-1 polypeptide chain of 110 amino acids is folded into characteristic beta-pleated sheet structure of an immunoglobulin variable domain (Fig 4). The amino acid sequence and the position of its disulphide bonds shows homology with an Ig domain. Integration into the membrane of the lipid bilayer is via a glycoposphatidylinositol (GPI) tail attached to the COOH-terminal residue. The apparent molecular weight of Thy 1 determined by SDS-polyacrylamide gel electrophoresis is between 24-29 kDa depending on species and tissue used as a source. The true molecular weight is 17.5 kDa. It is the presence of carbohydrate chains which make the molecule look larger. The Thy-1 gene contains four exons and is found on chromosome 9 in mouse and chromosome 11 in man. In mice, Thy-1 exists in two allelic forms, Thy-1.1 and Thy-1.2. A single amino acid interchange at residue 89 (arginine in Thy-1.1 and glutamine in Thy-1.2) is responsible for the two alloantigenic forms.

Thy-1 is a major surface component of mature neurons (except the primary olfactory neurons, Morris, 1985). However, during normal development it is excluded from axons until after they have finished growing (Xue *et al.*, 1990 and 1991; Xue and Morris, 1992). In their immature state, neurons express high levels of Thy-1 mRNA and Thy-1 protein is seen on their dendrites many days before Thy-1 appears on axons of mature neurons. There are two exceptions; one is the lack of expression of Thy-1 on olfactory neurons, the axons of which are able to grow into the adult CNS; another is that Thy-1 is also present on growing axons of sympathetic neurons which do not normally grow into the CNS and so do not encounter astrocytes. This suggests that Thy-1 is excluded during normal development, not from growing axons, but from axons growing in CNS. Developmentally regulated molecules such as Thy-1 may provide important

**FIG 4.** Model of Thy-1 in a lipid monolayer : the polypeptide chain is shown in a ribbon model, the glycan chain of glucosyl phosphatidyl inositol (GPI) anchor in space-filling format, and the lipids as stick models. The arrows represent strands of beta-pleated sheet which are joined by loop region. Carbohydrate chains cover much of front left and rear of molecule. Residue 89 (green) is either glutamine or arginine in mice, conferring the Thy-1.1/1.2 allelic antigenic determinant to the molecule.



clues in studying regenerative capacity of severed CNS axons in the adult mammalian system.

Early *in vitro* work showed that neurite growth from rat and mouse retinal ganglion cells (Lipton *et al.*, 1988), rat adrenal cells and PC12 cells (Mahanthappa *et al.*, 1987) all of which express Thy-1 on their surface, is enhanced if these cells are cultured on a substrate coated with antibodies against Thy-1. Evidence for the inhibitory effect of mature CNS derived glia on axonal growth was demonstrated when a Thy-1 negative neural cell line, NG115-401L was transfected with Thy-1, and the expression of Thy-1 inhibited neurite outgrowth by these cells on mature astrocytes but not on axon growth-promoting cells such as embryonic glia and Schwann cells (Tiveron *et al.*, 1992). It was suggested that Thy-1 directly initiates a neurite growth inhibitory signal across the membrane of the neural cell thus downregulating the growth of axons contacting mature astrocytes. Thy-1 negative (control) neurons extended neurites on mature astrocytes and growth promoting substrates with equal vigour. Direct contact between Thy-1 expressing cell and the astrocytes was needed to inhibit neurite outgrowth; cells located on plastic near the astrocytic monolayer extended their processes in an uninhibited fashion. Purified Thy-1 was shown to bind to astrocytes, whereupon it blocked their ability to inhibit neurite outgrowth. The hypothesis is that Thy-1 is the receptor molecule on the adult neuronal cell surface which interacts with some ligand on the astrocytic surface to inhibit neurite outgrowth (Morris, 1992). This evidence suggests that *in vivo*, Thy-1 interacts with astrocytes (which in normal animals do not themselves express Thy-1) (Morris *et al.*, 1985a) in the terminal fields to restrict further axonal growth and stabilise the pattern of connections formed.

Thy-1 molecule does not span the lipid bilayer yet the inhibitory signal is transmitted directly into the neural cell by Thy-1. It has been suggested that GPI anchors direct the proteins they tether to specific microdomains on the membrane,

where they function by accessing certain transmembrane signalling molecules (Lisanti *et al.*, 1994). Although the structure of Thy-1 and its capacity to act as signal transducer is well documented, the question remains as to which of its domains is involved in specific functions of the molecule, such as cell adhesion and neurite outgrowth or signal transduction. In experiments where transmembrane variant forms of Thy-1 were made by exchanging the region of DNA coding for the transient transmembrane anchor of Thy-1 with regions coding for the transmembrane and truncated cytoplasmic domains of CD8 and NCAM-140, the GPI anchor was found to be necessary for Thy-1 function. Stronger inhibition of neurite outgrowth was seen on astrocytes with the GPI form of Thy-1 than with the modulated transmembrane form. The transmembrane anchor directed Thy-1 to a different microdomain where it was functionally inactive (Tiveron *et al.*, 1994). The hypothesis is that Thy-1 interacts with two molecules, an astrocytic ligand and a neural transmembrane component of Thy-1 glycoprotein which mediates neurite growth inhibitory signal. Access to its transmembrane signalling partner is only possible when Thy-1 is in a GPI domain, and thus the transmembrane forms are inactive. It was recently reported that a particular domain of L1 is responsible for linkage of cell surface recognition during neurite outgrowth to signal transduction (Appel *et al.*, 1995).

## **VI                    USING DORSAL ROOT GANGLIA TO STUDY**

### **NEURON-ASTROCYTE INTERACTION MEDIATED**

#### **BY THY-1**

This project focuses on the involvement of Thy-1 in restricting axonal growth by primary dorsal root ganglia (DRG) cells on mature astrocyte monolayer.



Studies with neuronal cell lines *in vitro*, where neurite growth is less exuberant, showed inhibition of neurite outgrowth on astrocytes by Thy-1 as an all-or-nothing effect. Where primary neurons are concerned, down-regulation of growth following inhibition of one receptor system may be partial and not strongly evident due to the compensatory action of other receptor systems (Neugebauer *et al.*, 1988). Partial inhibition would be difficult to assay *in vivo*, but *in vitro* it may show up as an effect on rate of growth rather than an all-or-nothing effect. In order to assess the possible role of Thy-1 in restricting axonal growth over mature astrocytes, growth of Thy-1 positive DRG neurons derived from adult mice, was compared with growth of control adult sensory neurons derived from mice that lack Thy-1. This is achieved by using mice in which Thy-1 gene has been inactivated by homologous recombination (Thomas and Capecchi, 1987).

DRG neurons have been chosen for this study because they can be grown successfully *in vitro* from embryonic stages (when they lack Thy-1), to adult (when they express Thy-1). Their neurite outgrowth is axonal and not dendritic in character. DRG cells have been studied in great detail; their trophic factors are known and their initial growth and regeneration in mature animals is well documented (Scott, 1992).

## A THE DRGs

DRG neuronal precursors from the neural crest migrate to lie ventrolaterally to the spinal cord (LeDouarin and Smith, 1988). There is an anterior to posterior gradient of maturation in DRG development. The development and mitotic division of crest cells within the ganglion result in an increase in cell number which continues even after neural crest cells have ceased to emigrate from the neural tube. Several lines of experimental evidence show that the cells in these ganglia, both neuronal and non neuronal, develop from precursors originating in the neural crest (Le Douarin, 1988).

DRG cells reside in discrete ganglia which are recessed in bony cavities along the vertebral column. DRG ganglia are comprised of neurons with associated non-neuronal cells, satellite cells, and also Schwann cells and fibroblasts around axons. Within each ganglion a single axon leaves the DRG cell body and bifurcates. It has a peripheral branch that receives information from sensory receptor organs and a central branch that travels in the dorsal root on its way to the spinal cord or brain stem and transmits information centrally.

Every dorsal root ganglion contains over 20 functionally distinct types of DRG neurons that innervate different tissues. It has been suggested that sensory neurons innervating different organs have somata with significantly different sizes e.g., sensory neurons innervating heart and liver have the smallest somata size and those innervating feather follicles in the chick have the largest (Zhou *et al.*, 1994). The different sizes of DRG cells known in adult vertebrate correlate with function, cell birthdays, and histochemistry. Dorsal root ganglion sensory neurons have been divided into two histological types called "large light" (L), containing large amounts of neurofilament and "small dark" (S) containing fewer neurofilaments (Lawson *et al.*, 1984) on the basis of their staining properties under the light microscope and in electron microscopic studies (Willis and Coggeshall, 1992). The L neurons develop earlier than the S neurons. Yoshida described a linear relationship between cell size and axon diameter in mouse DRG cells filled with horseradish peroxidase (Yoshida and Matsuda, 1979 ).

*In vivo* (Johnson *et al.*, 1980) and *in vitro* (Lindsay, 1988) NGF is not continuously required as a survival factor for mature sensory neurons. NGF has been shown to regulate the response of adult DRG neuron to excitotoxin capsaicin (Winter *et al.*, 1988). Like NGF and BDNF, NT3 also promotes the survival of early DRG neurons in culture but differs from these neurotrophic factors in the particular set of sensory neurons it supports from rat and chicken embryos (Memberg and Hall, 1996; Lefcort *et*

*al.*, 1996). The receptors for the neuronal growth factors have been identified. Trk A tyrosin protein kinase is the high affinity receptor for NGF but also binds NT3, the Trk B tyrosine protein kinase is a receptor for BDNF and NT3 and Trk C tyrosine protein kinase receptor is a receptor for NT3. The Trk gene family may play an important role in the development and maintenance of specific cell types within the mammalian nervous system (Barbacid *et al.*, 1991). Among the neurotrophins only NGF has a major survival-promoting effect on neonatal DRG neurons in culture (Memberg and Hall, 1996). These findings suggest that many DRG neurons are responsive to multiple neurotrophins early in development before becoming more restricted in their survival requirements later on. Studies have shown that the loss of DRG neurons in *trkA*<sup>-/-</sup> ; *trkB*<sup>-/-</sup> double knock-out mice is not significantly greater than that occurring in *trkA*<sup>-/-</sup> mice (Minichiello *et al.*, 1995) suggesting that at least some DRG neurons are dependent on both TrkA and TrkB signalling pathways for survival. In DRG of *NT3*<sup>-/-</sup> and *TrkC*<sup>-/-</sup> mouse embryos, data indicated a reduction in the numbers of DRG neurons and an increase in the numbers of cells undergoing apoptosis. NT3 appears to play a role in controlling the proliferation of DRG precursor cells and is required for the survival of a proportion of early neurons (White *et al.*, 1996).

In the adult, even within the same ganglion DRG cells are remarkably diverse. Ranson (1912) observed that large cells give rise to myelinated axons and small cells to unmyelinated axons. Molecular differences between DRGs have now been established. These molecular features range from structural proteins of neurofilaments (Lawson and Waddell, 1991) to putative transmitter agents, such as glutamate, various peptides, and purines (Jessel and Dodd, 1986; Rustoni and Weinburg, 1989). Peptide distribution of Substance P and calcitonin gene-related peptide (CGRP) has been used widely to characterise DRG neurons (Hökfelt *et al.*, 1976; Price, 1985). Substance P immunoreactivity is largely confined to small and medium sized neurons (15-40  $\mu$ m in diameter) and CGRP was present in cells of all sizes including very large cells greater than 50-60  $\mu$ m in diameter (Lindsay *et al.*, 1989). Many molecular and structural features

are common to sets of neurons. CGRP peptide coexists with Substance P, somatostatin and galanin. A subpopulation of small DRG neurons do not contain measurable levels of peptides. Immunocytological and histochemical techniques have been used extensively to study the presence of peptides, enzymes and carbohydrate groups in DRG, while *in situ* hybridisation has allowed the study of specific mRNAs. Primary sensory neurons are a unique example of coexisting systems expressing both classical transmitters and peptides as well as other messengers such as nitric oxide (Höckfelt, 1991). The huge diversity of DRG neurons means that molecule of interest may not be expressed identically, or at equivalent levels by all subsets of DRGs. This presents problems in terms of analysis.

## B     THE ASSAY

Change in neurite outgrowth of Thy-1 positive and Thy-1 negative DRG neurons was measured by recording total length of neurites that grew over astrocytes, compared with the degree of axonal growth displayed by neurons plated on control substrate.

Basic methods have been established which allow the maintenance of adult and embryonic dissociated DRG cells in culture (Lindsay, 1988a and Bedi *et al.*, 1992). I have developed a culture system of DRG cells which yield optimally viable DRG cells with improved neurite outgrowth.

I have been able to show that the axonal growth of adult DRG neurons expressing Thy-1 on the cell surface was mildly inhibited when compared to Thy-1 deficient neurons. I anticipated that any effect of Thy-1 on the vigorous growth of adult sensory neurons would only be partial and that the difference in rate and pattern of growth between the Thy-1 negative and positive axons would become important factors in judging inhibition. Mild effects due to the action of any one receptor system have been

seen with other molecules (N-cadherin, NCAM and integrins) (Neugebauer *et al.*, 1988; Tomaselli *et al.*, 1988) implying that several factors are involved in regulation of neurite extension over mature astrocytes. Complex regulation needs a more physiological *in vitro* assay, e.g., at least a three-dimensional system (Fawcett *et al.*, 1989a) which I have developed. The three-dimensional system may provide a more accurate means for study of a heterogeneous population of cells such as DRGs where the onset of neurite extension is different for the various populations of cells. This assay system therefore, takes into consideration the whole array of cells in a heterogeneous population of cells and includes the late starters as well as the early starters.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 MATERIALS

##### 2.1.1 TISSUE CULTURE REAGENTS

###### 2.1. 1-1 BUFFERS, DIGESTION AND DISSOCIATION AGENTS

###### Phosphate buffered saline (Mg<sup>2+</sup> and Ca<sup>2+</sup> free)

Per litre of distilled water :

NaCl 8.00 gm

KCl 0.20 gm

Na<sub>2</sub>HPO<sub>4</sub> 1.15 gm

KH<sub>2</sub>PO<sub>4</sub> 0.20 gm

pH neutral. Sterilised by autoclaving.

###### Trypsin versene

NaCl 0.800 gm

KCl 0.020 gm

Na<sub>2</sub>HPO<sub>4</sub> 0.115 gm

KH<sub>2</sub>PO<sub>4</sub> 0.020 gm

EDTA 0.010 gm

Trypsin 0.125 gm

Phenol red 0.001 gm

Distilled H<sub>2</sub>O 100 ml

pH adjusted to 7.8. Filter sterilised.

###### Collagenase [type II derived from *Clostridium histolyticum* (Sigma. UK)]

75 mg dissolved in 50 ml MEM (stock at 1.5mg/ml).

Filter sterilised. Used at 0.15%

Trypsin-99%protein (Worthington Biochemical Corporation. USA)  
0.5 gm Trypsin dissolved in 50 ml PBS-CM. Filter sterilised.  
Used at 0.1%.

## 2.1. 1-2      CELL CULTURE SUPPLEMENTS

### Antibiotics+glutamine

Penicillin (Gibco.UK)	0.60 gm
Streptomycine (Gibco)	1.00 gm
L-glutamine	2.92 gm
Distilled H <sub>2</sub> O	100 ml

pH neutral. Membrane filtered to sterilise.

### Proline (BDH)

Prepared in MEM at a concentration of 4 g/l. Filter sterilised. Used at 1:100 (40 mg/l).

### Thymidine (Sigma.)

Made up in MEM at a concentration of 73 mg/l.  
Sterilised using 0.4 micron filter unit. Final concentration 0.73 mg/l

### Glycine (BDH.)

750 mg/l dissolved in MEM and sterilised by filtration. Used at 1:100 (7.5 mg/l).

### Hypoxanthine (Sigma)

Dissolved at a concentration of 410 mg/l in distilled water after solublising in hydrochloric acid. Diluted 1:100. (4.10 mg/l)

### Cadmium Chloride (Sigma.)

1 mM solution was prepared in MEM. Final concentration 100 nm.

### 2.1. 1-3      GROWTH FACTOR

Nerve Growth Factor (NGF-2.5S. Serotec Ltd. U. K.)

10  $\mu$ g dissolved in 100  $\mu$ l MEM medium. Used at 75 ng/ml.

### 2.1. 1-4      CELL ATTACHMENT FACTORS

Laminin-from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma.).

1 mg/ml in Tris buffer. Diluted to 5  $\mu$ g/ml in PBS-CM.

Collagen

Collagen derived from rat tendons was at a concentration of approximately 1 mg/ml in glacial Acetic acid at pH4.0.

BUFFER MIX: 10 x BMEM (Gibco) pH 4.0      900  $\mu$ l

                         Sterile H<sub>2</sub>O      900  $\mu$ l

                         7.5% NaHCO<sub>3</sub>      250  $\mu$ l

225  $\mu$ l of buffer mix added to 900  $\mu$ l rat tail collagen.

### 2.1. 1-5      MISCELLANEOUS REAGENTS

Bovine Serum Albumin (Sigma) for Tissue culture

Bovine serum albumin 12.0 gm dissolved in 100 ml MEM.

Filter sterilised.

Cytosine Arabinoside-AraC (Sigma)

2.8 mg per ml solution made in sterile distilled water. Used at 10<sup>-5</sup>M.

Deoxyribonuclease-DNAse (Sigma)

5 mg per ml dissolved in PBS-CM and filter sterilised. Diluted 1:1000 for use.



Fluorescein di-acetate (Sigma)

5 mg/ml stock solution made in acetone and stored at -20°C.

Diluted to 5 µg/ml in PBS for use.

Trypan Blue solution (0.4%) (Sigma. UK)

Diluted 1:2 in MEM.

## 2.1.2

## IMMUNOCYTOLOGICAL MATERIALS

### 2.1. 2-1

### FIXATIVES

1% Paraformaldehyde (BDH)

Paraformaldehyde      1 gm

PBS-CM                      100 ml

pH adjusted to 7.5 using 1M NaOH.

Ethanol-Acetic acid

95% ethanol                95 ml

glacial acetic acid        0.5 ml

### 2.1. 2-2

### ANTIBODIES

Anti Glial Fibrillary Acidic Protein (GFAP) (Dako Patts. Denmark)

Rabbit anti cow GFAP used at a concentration of 1:200

Anti-Thy-1.2

30.H.12 rat anti Thy 1.2 monoclonal antibody (Ledbetter and Herzenberg,1979).

Used 1:30 dilution of tissue culture supernatant in the same buffer.

Anti-Thy-1.1 (OX 7)

Monoclonal antibody OX 7 (Mason and Williams, 1980). Used at a concentration of 1:1000.

Anti-neurofilament antibody (RT97)

RT97 labels a phosphorylated epitope on the 200 kDa subunit of neurofilaments.(Anderton *et al* , 1982). Monoclonal antibody was obtained by growing RT97 hybridoma cells and using the cell culture supernatant at a concentration of 1:400.

Anti-tubulin antibody (TuJ 1) (a gift from Tony Frankfurter)

TuJ 1-antibody raised in rat against anti-class III  $\beta$ -tubulin at a concentration of 1:800

Anti-GAP-43 antibody (gift from Graham Wilkins)

Anti-growth-associated antibody raised in rabbit.  
Used at 1:2000

Anti-MAG antibody

Antibody raised in the mouse against myelin associated glycoprotein.  
MAG 513 Hybridoma cell line supernatant used neat.

Anti-Substance P antibody (Amersham International plc. U.K)

Antiserum to Substance P raised in rabbits. Used at 1:200.

Anti-CGRP antibody (Amersham International plc. U.K.)

Antibody to calcitonin gene related peptide. For use diluted 1:100.

Anti-fibronectin rabbit polyclonal antibody (Dako. Denmark)

Diluted antibody 1:500 in antibody diluent for use.

Anti-CSPG (chondroitin sulphate proteoglycan) (Sigma. U.K)

Mouse monoclonal used at 1:200.

Rabbit anti mouse IgG (RAM IgG) (Dr R Morris)

Unconjugated affinity purified F(ab')<sub>2</sub>. Used at 1:1000

Rabbit anti rat IgG (RAR-FITC) (Dako. Denmark)

FITC conjugated immunoglobulin at a concentration of 1:200.

Sheep anti Rabbit IgG (SAR-FITC) (Dr R Morris)

Affinity purified F(ab')<sub>2</sub> anti-rabbit F(ab')<sub>2</sub> conjugated to FITC.

Concentration for use 1:200 or 10 µg/ml

Rabbit anti Mouse IgG (RAM-TRITC) (Dako. Denmark)

TRITC- conjugated antibody used at 1:50

### 2.1. 2-3 MISCELLANEOUS REAGENTS

bisBenzimide

bisBenzimide (Höchst) (Sigma) dissolved in dimethyl sulphoxide to 10 mg/ml and was diluted 1:1000 in PBS for use.

### 2.1. 2-4 BLOCKING AGENTS AND ANTIBODY DILUENTS

Rabbit serum

Used for blocking to reduce non-specific binding at a concentration of 1:20

<u>BSA</u>	<u>1% BSA</u>	<u>10% BSA</u>
BSA	1 gm	10 gm
PBS	100 ml	100 ml
Sodium Azide(5%)	200 µl	200 µl

1% BSA was used as antibody blocking agent for reducing non-specific binding and 10% BSA was utilised as antibody diluent.

### 2.1. 2-5 MOUNTANT

Vectashield® (Vector laboratories. USA.) water soluble mounting medium prevented rapid photobleaching.

### **2.1.3 ANIMALS**

Young adult mice (27-30 days; age matched to within 1 day in each experiment) of the 129/Sv strain, either wild type (Thy-1 positive) or knockout (Thy-1 negative) were used.

### **2.1.4 GENOMIC DNA ANALYSIS MATERIALS**

#### **2.1. 4-1 DNA EXTRACTION**

##### **Tail Mix**

50 mM Tris pH 8.0 (1 M)	2.5 ml
100 mM EDTA (0.5 M)	10 ml
100 mM NaCl (5 M)	1 ml
1% SDS (20%)	2.5 ml
H <sub>2</sub> O	34 ml

##### **Proteinase K** (Sigma. UK)

Prepared as a stock of 10 mg/ml.

Final concentration for use 3.5 mg/ml.

##### **Ribonuclease (RNAse)** (Boehringer Mannheim. UK)

Stock at 10 mg/ml and use at 1.4 mg/ml.

##### **Phenol/Chloroform** (Appligene. France; BDH;Sigma)

Phenol pH 8.0	25 ml
Chloroform	24 ml
Isoamyl alcohol (IAA)	1 ml

##### **Chloroform/IAA** (iso-amylalcohol)

Chloroform	24 ml
IAA	1 ml

#### DNA precipitation cocktail

NaCl (5 M)	30 $\mu$ l
Isopropanol	0.6 vol (430 $\mu$ l)
Sample DNA	300 $\mu$ l

### 2.1. 4-2      DNA DIGESTION AND SOUTHERN ANALYSIS

Restriction enzymes and restriction buffers were purchased from Böehringer Mannheim

#### Digest Mix

In 60 $\mu$ l	DNA (11 $\mu$ g)+H <sub>2</sub> O
	6 $\mu$ l Restriction buffer
	50 U enzyme (1.25 $\mu$ l of 40 Units/ $\mu$ l <i>Bam</i> H1)

### 2.1. 4-3      GEL ELECTROPHORESIS

#### Agar 0.8%

Electrophoresis grade Agar	0.8 gm
TAE buffer	100 ml

DNA Lamda Marker (Lamda/RI 125 ng/ml=16  $\mu$ l)

Electrophoresis buffer TAE

Ethidium bromide.

#### Depurination Buffer

HCl	5.4 ml
H <sub>2</sub> O	250 ml

#### Denaturation buffer

0.5M NaOH (10 M)	12.5 ml
1.5M NaCl (5 M)	75.0 ml
H <sub>2</sub> O	162.5 ml

#### Wetting solution

0.5 M Tris-Cl (1 M) pH7.	450 ml
1.5 M NaCl (5 M)	30 ml
H <sub>2</sub> O	20 ml

### 2.1. 4-4

#### PRE-HYBRIDISATION AND HYBRIDISATION MIX

##### Pre-Hybridisation Mix

3 x SSC	3.75 ml of 20X SSC
0.1% SDS	125 $\mu$ l of 20% SDS
Denhardt's	2.5 ml of 10X solution
50 $\mu$ g/ml ssDNA	125 $\mu$ l of 10mg/ml stock
H <sub>2</sub> O	18.5 ml

##### Isotopically labelled probe in Hybridisation Mix

Thy-1 ( <i>Apa</i> 1) probe + H <sub>2</sub> O	4 $\mu$ l (approx 100 ng DNA)
dGCT mix	2 $\mu$ l
DNA-polymerase I enzyme	1 $\mu$ l
<sup>32</sup> P-dATP (Amersham.UK.)	3 $\mu$ l
3 x SSC	1.2 ml of 20X SSC
0.1 % SDS	40 $\mu$ l of 20% SDS
Denhardt's	800 $\mu$ l 10X stock solution
50 $\mu$ g/ml ssDNA	40 $\mu$ l of 10mg/ml stock
Dextran sulphate	5 ml of 10% solution

##### Sephadex column

EDTA	300 $\mu$ l
G50 Sephadex	1 ml (packed volume)

##### Wash buffers

3 x SSC+0.1% SDS in 250 ml
0.2 x SSC+0.1% SDS in 250 ml

A Zeiss Axiovert microscope linked to a Hamamatsu C3077 CCD camera (Hamamatsu Photonics K.K. Japan) and Fluorovision software (ImproVision, Coventry, U.K.) was used for image analysis.

## **2.2**

## **METHODS**

### **2.2.1**

### **ANIMALS**

Knockout mice were generated in the United States from inbred strain 129/Sv embryonic stem (ES) cells in which both copies of Thy-1 gene on chromosome 9 (Blankenhorn and Douglas, 1972) were inactivated by homologous recombination (courtesy of Colin Stewart, Roche Institute of Molecular Biology, Nutley N.J. and Jack Silver, North Shore University Hospital, Manhasset, N.Y.). Briefly, Thy-1 gene was inactivated by insertion of a neomycin resistance element at a *Bst*E11 restriction site to disrupt the Thy-1 gene at the beginning of the coding region of the mature Thy-1 protein, just 3 amino acids from the amino terminus (Fig 5). Insertion of the ES cells into fertilised blastocytes produced chimeric mice that were bred to C57Bl/6 mice, introducing hybrid vigour in the F1 generation. Pups of the first F1 generation, found to have a copy of the inactivated gene were interbred to establish the Thy-1 knockout (Ko) line. No Thy-1 protein can be detected in these animals using a variety of immunological assays, with both polyclonal and monoclonal antibodies. Animals were bred and maintained in our transgenic facilities. Genomic DNA was analysed using the Southern technique.



## **2.2.2 GENOTYPING BY SOUTHERN ANALYSIS**

### **2.2. 2-1 DNA EXTRACTION**

5-8mm of 8-10 day old mouse tail was cut and preserved in 700  $\mu$ l tail mix containing 50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl, and 1% SDS. Proteinase K to a concentration of 3.5 mg /ml was added and proteins were digested by incubating at 55°C overnight. RNase (1.4 mg/ml) was added and the tails were incubated for 30 minutes at 37°C.

A mixture of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA mix and shaken gently on a platform for 15 minutes. Centrifugation at 15000 rpm for 15 minutes separated the aqueous phase and organic phase. Using a wide bore pipette, the viscous aqueous phase (top) was transferred to a fresh 1.5ml Eppendorf tube. The extraction was repeated once more with phenol:chloroform:IAA and a finally with chloroform : IAA (24:1). After the final extraction, the aqueous phase was collected and DNA precipitation was performed by shaking the aqueous phase in high salt concentration and 0.6 volume of isopropyl alcohol. Intact DNA was removed using a hook shaped glass rod. DNA was air dried after dipping in 70% ethanol to remove any traces of water. DNA was dissolved at 37°C in a known volume of sterile distilled water and concentration of DNA was determined by reading optical density at a wavelength of 260nm on a spectrophotometer (Hitachi, Japan).

## 2.2. 2-2      RESTRICTION ENZYME DIGESTION and GEL ELECTROPHORESIS

11 $\mu$ g of DNA was mixed with restriction buffer and restriction enzyme *Bam*H1 at 37°C for 6-8 hours to cleave the double-stranded DNA into discrete pieces.

A 0.8 % agar mini gel was prepared, and cleaved DNA was mixed with loading buffer and ethidium bromide (5  $\mu$ g/ml) and loaded into wells. Migration of DNA was allowed to proceed for 40 min at a voltage of 60 V. A Polaroid picture was taken under UV light source to check efficiency of digestion, the physical state and concentration of DNA.

## 2.2. 2-3      SOUTHERN BLOTTING

Overnight electrophoresis in 1% agar at a running voltage of 40 V was used to separate, identify and purify DNA fragments. Efficiency of migration of DNA was assessed using molecular size markers and visualised using a UV light source. Depurination for 20 minutes and denaturation for 40 minutes was carried out under high alkaline conditions to separate DNA strands in the gel. The gel was placed in contact with a reservoir containing 20x SSC solution and overlaid with nitrocellulose paper which is in turn overlaid with paper towels and weighed down. Buffer from the reservoir flows through the gel, carrying with it the DNA fragments. Transfer was allowed to proceed for 24 hours. Single stranded DNA was thus bound irreversibly to Amersham Hybond nitrocellulose filter and was cross-linked by exposure to UV light.

Radiolabelled DNA probe was prepared using DNA polymerase I to replace unlabelled nucleotides in a double stranded DNA with alpha  $^{32}\text{P}$  labelled nucleotides. Free unlabelled d GTP, CTP and  $^{32}\text{P}$ -dATP nucleotides were incorporated into probe DNA (100 ng) in the presence of DNA polymerase I for 1 hour at 18°C. The nick translated DNA was separated from un-incorporated dNTPs by centrifugation through a small column of Sephadex G50. The  $^{32}\text{P}$  labelled probe was exposed to a temperature of 100°C to denature the probe and then quickly put on ice for 1 minute to maintain a large percentage of single stranded DNA.

The nitrocellulose filter bearing the nucleic acids was pre-hybridised at 65°C for 2 hours to suppress background hybridisation in a mix containing 5 x Denhardt's reagent , 0.5%SDS, and 100 $\mu\text{g/ml}$  sonicated salmon sperm DNA.

The labelled *Apa* 1 probe was allowed to hybridise overnight at 65°C to the target (*Bam*H1 digested DNA) in 7.5ml hybridisation mix. Stringency washes at 65°C, twice in 3 x SSC for 15min and 0.2 x SSC for 15 min, were sufficient to remove unbound nucleotides.

Isotypically labelled probe, bound to its target was detected by autoradiography after exposure on X-ray film for 48 hours at -70°C. Thy-1 gene orientation and insertion was confirmed. Genotyped homozygous Thy-1<sup>-/-</sup> and Thy-1<sup>+/+</sup> mice were selected for experiments.

**2.2. 3****PREPARATION OF SUBSTRATE FOR AXONAL****GROWTH STUDIES**

Laminin and mature rat astrocytes were used as substrate for studies of neurite outgrowth from sensory neurons in primary culture. A three-dimensional substrate was developed to study the contribution of Thy-1 in inhibition of neurite outgrowth.

**2.2. 3-1****LAMININ SUBSTRATE**

Primaria® surface modified 35mm tissue culture dishes were coated for 2 hours with laminin dissolved in PBS-CM at a concentration of 5 µg/ml. Before seeding with dissociated DRG neurons, the laminin coated dishes were washed once with PBS-CM.

Astrocytes were prepared from *corpus callosum* of 5-8 day old rat pups. The brain was aseptically dissected out into S-MEMF medium. *Corpus callosum* was exposed by cutting away the cortex in very thin slightly sloping horizontal sections. The callosum was cleaned to remove meninges and excess tissue and all the callosa were collected in a test tube after being chopped into pieces of approximately 0.5 mm<sup>2</sup>. The tissue was washed once with PBS-CM. Tissue was dissociated by incubating it in 0.1% trypsin at 37°C for 10 minutes. The tissue was then resuspended in S-MEMF medium and centrifuged at 800 rpm for 5 minutes. The pellet was resuspended by gently triturating clumps with a fine bore pipette. The cell suspension was seeded to a 25 cm<sup>2</sup> flask. Cells were cultured in S-MEMF medium. After 8-10 days in culture, the vessel was covered with a monolayer of heterogeneous cell populations. Loosely attached, rapidly dividing non-astrocytic cells were discarded by mechanically shaking the flask (70 shakes/minute) at 37°C overnight. To obtain a purer astrocyte culture, the rapidly dividing non-astrocytic cell population was killed by incubating in S-MEMF medium containing cytosine arabinoside (AraC) at a final concentration of 10<sup>-5</sup> M. Astrocytes were maintained in S-MEM medium to which 10% foetal calf serum was added. Cells were kept at 37°C in a humidified 95% air/5% CO<sub>2</sub> atmosphere. Astrocyte monolayers used for neurite outgrowth studies were 97-99% positive for glial fibrillary acidic protein (GFAP) and were used after they had been in culture from between 2-6 months. For neurite outgrowth experiments, astrocytes were seeded on to 35 mm tissue culture dishes at a density of 3 x 10<sup>4</sup> cells per dish and they were used 7-9 days later when a confluent flattened monolayer was formed.

Astrocyte cultures were monitored for mycoplasma contamination (culture broth screening courtesy of Ms A. Leach, Biological Services) and purity of cell type (by GFAP staining).

Cell culture inserts (24.5 mm) were coated by layering 1ml of collagen onto the microporous membrane. Excess collagen was removed by tilting the insert and the collagen was allowed to dry at room temperature. Collagen coated inserts were equilibrated in MEM for two hours. Astrocytes at a concentration of  $3 \times 10^5$  cells per ml in S-MEMF were plated so that a confluent monolayer of astrocytes was formed after a period of 24 hours in a humidified CO<sub>2</sub> incubator at 37°C.

EXPRESSING SUBSTRATE

Control (COS) R2C cells-the non-MAG expressing cells were maintained in MEMF containing proline (40 mg/l). Differentiation medium for untransfected cells was MEMF containing proline (40 mg/l), thymidine (0.73 mg/l), glycine (7.5 mg/l) and hypoxanthine (4.1 mg/l). MAG transfected COS cells were plated so that a confluent monolayer was established over a period of 24 hours. Transfected cells were cultured in MEM containing 10% FCS, proline (40 mg/l), thymidine (0.73 mg/l) and glycine (7.5 mg/l). High expression of MAG by COS cells was achieved by addition of 100 nM cadmium chloride and 10 % dialysed FCS to the cells for 24 hours prior to assay.

**2.2.4-1**      **PREPARATION OF DISSOCIATED DORSAL ROOT GANGLION**  
**NEURONS**

Adult mice were sacrificed using cervical dislocation and embryonic material was derived from pregnant mice killed by cervical dislocation and subsequent delivery of embryos by Caesarean section.

Adult DRGs were removed by making a dorsal-midline incision along the body from cervical to lumbar level, retracting the skin to expose the vertebral column which was cut centrally to expose the spinal cord. Using a dissecting microscope DRGs were removed from their recessed bony cavities and collected in pH stable CIB medium. Embryonic DRGs were removed by dissecting along the neuraxis from cervical to lumbar levels with fine jeweller's forceps under a microscope.

The method of Lindsay (1989) was modified to optimise the yield of singly dissociated, viable and vigorously growing neurons. DRG were dissected out aseptically from adult (P27-30) mice and collected in 500  $\mu$ l of CIB medium. This provided healthy well oxygenated ganglia that were carefully excised under a dissecting microscope to remove peripheral and central trunks of each ganglion. The 'cleaned' ganglia were finely chopped and collected in S-MEMF medium. Using a pipette the S-MEMF medium was removed and replaced with 0.15% collagenase to disaggregate the tissue. Adult tissue was treated with collagenase at 37°C for 1.3 hour and embryonic tissue was treated with collagenase for 45 minutes. The collagenase was limited to a mere film covering the tissue. Collagenase was renewed twice and the tissue was agitated by hand every 15

minutes. Ganglia were washed once with PBS-CM and adult ganglia were treated with 0.125% trypsin for 30 minutes at 37°C and embryonic ganglia were incubated in trypsin for 15 minutes. S-MEMF was then added to inactivate the trypsin and the neurons were pelleted by centrifugation at 800 rpm for 5 minutes. The pellet was resuspended in S-MEMF containing DNase at a concentration of 5µg/ml to prevent aggregation by DNA released from dead cells. The cell suspension was triturated gently 6-8 times by passing through a fine bore fire polished pipette then layered onto a 12% BSA cushion (2 ml in a 15 mm x 120 mm tube) and centrifuged for 9 minutes at 200 g (1053 rpm). The pellet was resuspended in S-MEMF. DRG cells when examined under a microscope were phase-bright ranging in cell diameter from 15-70 microns.

Viability of the cells was assessed using trypan blue and confirmed using fluorescein diacetate (FDA).

For each experiment, ganglia were removed from a single experimental and control animal, and the order in which the animals were sacrificed was varied.

#### 2.2. 4-2      GROWTH OF DRG CELLS ON LAMININ

Dissociated DRG cells were plated on laminin at a concentration of approximately 300 cells per 35 mm dish. Cells were incubated in a humidified incubator at 37°C in S-MEMF and supplements which included nerve growth factor (70ng/ml) DRG cells were fixed in 1% paraformaldehyde at specific time intervals.



AMONOLAYER

DRG cells at a concentration of 300 cells were seeded onto a 35 mm dish of monolayer of mature astrocytes. Cells were cultured in S-MEMF containing NGF (70 ng/ml). Fixation using 1% paraformaldehyde was carried out after a known period in culture.

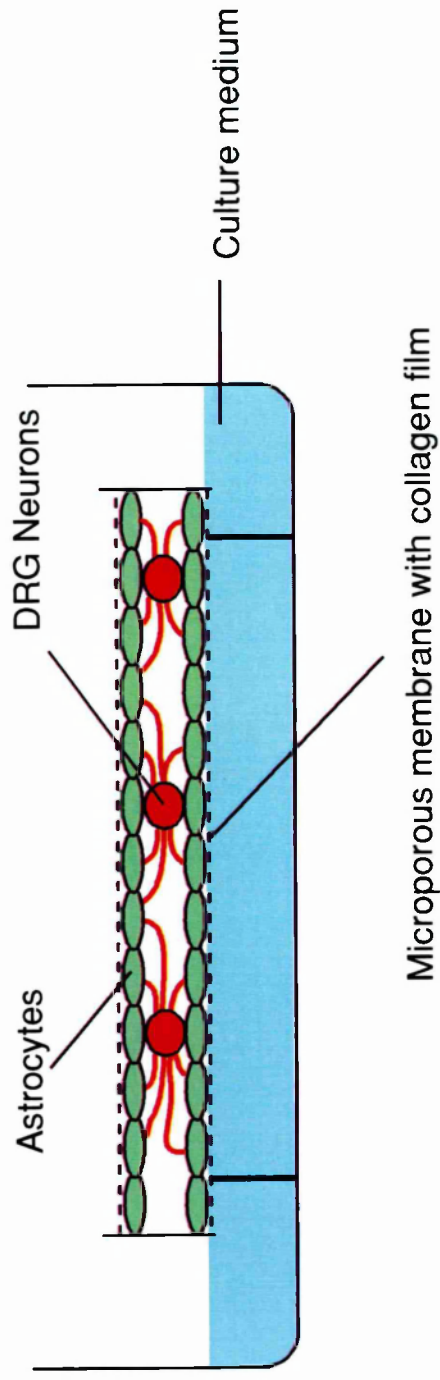
BCOLLAGEN-ASTROCYTE SANDWICH

To study growth of neurites in a system which more closely resembles the *in vivo* situation a three-dimensional assay was developed (Fig 5). Approximately 800 dissociated DRG cells in minimum volume of medium were plated onto the astrocyte-collagen substrate and allowed to attach for 2 hours. A second astrocyte-collagen layer was carefully placed over the attached DRG cells on the bottom astrocyte-collagen layer. The astrocyte-collagen culture sits on microporous membrane filter in an organ culture dish. This assembly was in contact with S-MEMF containing 70 ng/ml NGF. Cells were incubated at 37°C in a humidified incubator and immunolabelled for visualisation of neurite outgrowth at known time intervals.

2.2.4-4GROWTH OF DRG CELLS ON MAG EXPRESSING COSAND R2C CONTROL CELLS

DRG cells were plated at 800 cells/35mm dish on confluent R2C control, or MAG expressing, COS cells for neurite outgrowth studies.

**FIG 5.** Three-dimensional culture system. DRG neurons are sandwiched between two astrocyte monolayers growing on a film of collagen on a microporous membrane. The insert vessel is in contact with the culture medium. The culture dish is covered and the cells are maintained at 37°C.



## 2.2. 5            IMMUNOLABELLING

### 2.2. 5-1            IDENTIFYING THY-1 POSITIVE CELLS

Rat monoclonal 30H12 (Ledbetter and Herzenberg, 1979) was used to detect the Thy1.2 determinant on mouse DRG cells.

Living DRG cells were washed with PBS and incubated in 1:30 dilution of 30H12 tissue culture supernatant at room temperature for 40 minutes. To reduce non-specific binding, the cells were incubated in 1% BSA for 5 minutes before adding FITC-conjugated rabbit anti rat IgG secondary antibody. The cells were left in the secondary antibody for 40 minutes at room temperature, and then washed with 1% BSA for 10 minutes before fixing in 1% paraformaldehyde for 1 hour and mounted for observation.

### 2.2. 5-2            IMMUNOLOGICAL LABELLING OF NEURITE OUTGROWTH

RT97, a monoclonal antibody against the phosphorylated form of the 200 kDa neurofilament subunit (Anderton *et al* , 1982) and TuJ 1, the anti-class III  $\beta$ -tubulin (Moody *et al.* 1989) were used together to label all fibre outgrowth from DRG neurons.

DRG cells were fixed in 1% paraformaldehyde for 1 hour and then washed with PBS. To perforate the cell membrane, the DRG cells were incubated in 0.1% Triton X-100 for 10 minutes. After a wash in PBS, primary antibody was applied for 1 hour (RT97

at a dilution of 1:400 and TuJ 1 at 1:800 dilution) at room temperature. After a wash in 1% BSA, the cells were incubated for a further hour at room temperature in unconjugated F(ab')<sub>2</sub> rabbit anti mouse IgG (1:1000). After two 5 minute washes in 1% BSA the cells were labelled with affinity purified FITC-sheep anti rabbit (10 µg/ml) for 1 hour at room temperature. The cells were mounted after washing for 10 minutes in 1% BSA, 3 minutes in PBS and fixing for 10 minutes in 1% paraformaldehyde.

## 2.2. 5-3      IMMUNOCYTOCHEMISTRY OF ASTROCYTES

### GFAP LABEL

Astrocytes were fixed and perforated for 10 minutes in a mixture of 5% acetic acid and 95% ethanol on ice, washed twice with PBS then labelled for 40 minutes at room temperature with rabbit anti-GFAP antibody and then washed in 1% BSA for 10 minutes. They were then incubated in FITC-sheep anti rabbit (1:50) for 40 minutes at room temperature and then washed in 1% BSA for 10 minutes and then PBS for 3 minutes. The cells were then incubated with the nuclear stain bis Benzimide for 3 minutes. Cells were washed briefly in PBS and mounted for observation.

### FIBRONECTIN LABEL

Sub confluent astrocytes were fixed in 4% paraformaldehyde and washed in PBS. Cells were treated with 0.1% triton for 10 minutes and then cells were incubated in anti-fibronectin antibody for 40 minutes at room temperature. After two washes in 1% BSA, the cells were incubated in anti-rabbit FITC for 30 minutes. Cells were labelled with H $\ddot{o}$ chst dye and mounted.

## CSPG LABEL

Fixed and perforated cells were incubated in the mouse monoclonal anti-CSPG antibody for 1 hour and then labelled with a TRITC conjugated secondary antibody for 30 minutes at room temperature. Cell nuclei were visualised using Hoechst dye.

## THY-1 LABEL

Thy-1.1 status of cells was assessed using OX7 antibody which was applied to live cells for 30 min at 37°C. After short incubations in 1% BSA, the cells were fixed in 4% paraformaldehyde and labelled with TRITC conjugated anti mouse antibody for 30 minutes. After labelling cells with Hoechst nuclear dye, the dishes were mounted.

## 2.2. 5-4      MAG and GAP-43 LABELLING OF COS CELLS and R2C

### CONTROL CELLS

After fixation, R2C control and MAG expressing COS cells were permeabilised with 0.1% Triton X-100 for 10 minutes and GAP 43 (1:20) was added for 30 minutes followed by 2 x 5 minute washes in 1% BSA and then a 1hr incubation with the secondary antibody sheep anti rabbit FITC to label the DRG neurite growth. Cells were blocked for 30 minutes with rabbit serum and then incubated for 1 hour with anti-MAG antibody (MAG 513 hybridoma supernatant). After 2 x 5 minute washes in 1 % BSA, MAG positive cells were visualised with rhodamine rabbit anti-mouse IgG (1hr diluted 1:50).

SUBSTANCE P and CGRP

DRG cells plated on laminin were fixed in 1% paraformaldehyde and labelled for calcitonin gene related protein (CGRP) (1:100) or Substance P (1:200) for 1 hour at room temperature. Treatment in 1% BSA for 5 minutes and subsequent labelling with sheep anti-rabbit IgG (1:200) identified subsets of DRG cells.

**2.2. 6.**      **VIABILITY TESTS**2.2.6-1      FLUORESCCEIN DI-ACETATE

DRG cells in MEMF were washed and resuspended to a single cell suspension in MEM. Fluorescein di-acetate diluted to a concentration of 5  $\mu$ g/ml was added to the cells for 20 minutes. After two washes in MEM the labelled (live-green) cells were visualised with UV optics.

2.2.6-2      TRYPAN BLUE TEST

0.2% trypan blue solution in MEM was added to a culture dish of astrocytes with DRG cells and incubated for 10 minutes. After two 5 minute washes in MEM, it was possible to distinguish dead DRG cells (labelled blue).

DRG cells which were labelled with a fluorescent antibody were visualised under fluorescence microscope. A Hamamatsu CCD camera captured the fluorescent image of each cell to be analysed and displayed its image on a visual display monitor. In general, cells tended to be at a higher density in the centre of the culture dishes, and here their neurites overlapped and could not be measured accurately. Cells for measurement were chosen randomly. Bias in favour of choosing cells with a simple neurite outgrowth was not present. Total neurite length was measured of 50 cells (for the morphologically simpler embryonic DRG neurons I was able to measure 100 neurons per dish) at each time point. For each cell with its neurite network, we captured and enhanced immunofluorescent profile on the 'Fluovision' software and traced in by hand very fine or weakly labelled neurites by comparing with the real cell by direct observation and excluding areas of high background fluorescence and the cell body. The total neurite outgrowth was then calculated by the image analysis system.

For each experimental variable analysis of variance was calculated using the SuperAnova computer software.



## **CHAPTER 3**

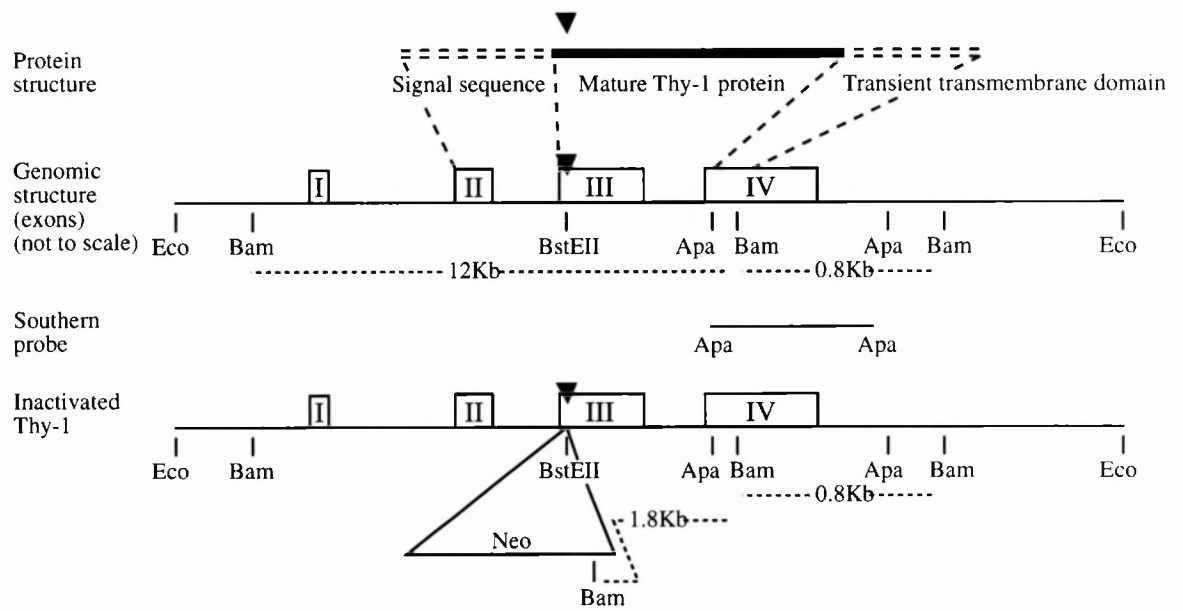
### **RESULTS**

#### **3. 1. IDENTIFICATION OF INACTIVATED THY-1 GENE**

Both copies of Thy-1 gene on chromosome 9 were inactivated (by Colin Stewart, Roche Institute, Nutley, New Jersey.) in embryonic stem (ES) cells of 129/Sv strain by homologous recombination. Neomycin resistance element at the *Bst* EII restriction site was inserted to disrupt the Thy-1 gene at the beginning of the coding region of the mature Thy-1 protein just 3 amino acids from the amino terminus (Fig 6).

The ES cells were kept for several months in culture and rare cells twice selected by G418 selection to obtain clones in which both copies of the Thy-1 gene were inactivated. Insertion of the ES clone with inactivated Thy-1 gene into fertilised blastocysts produced chimeric mice that were bred to C57Bl/6 mice introducing hybrid vigour in the F1 generation. Pups of the first F1 generation found to have a copy of the inactivated Thy-1 gene were interbred to obtain the first recombinant generation, which were sent to us at NIMR, Mill Hill. Initially, Thy-1<sup>-/-</sup> mice from this generation were bred to produce a Thy-1<sup>-/-</sup> line that was compared to normal (Thy-1<sup>+/+</sup>) 129/Sv mice. Mice of these two lines differed from each other in a number of striking ways (e.g. the Thy-1<sup>-/-</sup> mice had a distinctive lack of normal motor co-ordination of their rear limbs and tails; Thy-1<sup>-/-</sup> mice were markedly aggressive, and males were 50% heavier by 1 year of life than Thy-1<sup>+/+</sup> males).

**FIG 6.** Schematic diagram to show the position of the neomycin insert that disrupts the Thy-1 gene. Diagram shows the protein structure, genomic structure and the inactivated gene. Neomycin (Neo) resistant element has *Bam* H1 site 0.4kB from its 3' end, and is inserted at the *Bst* EII site shown. For analysis, the *Apa* 1 fragment was used to probe the DNA.



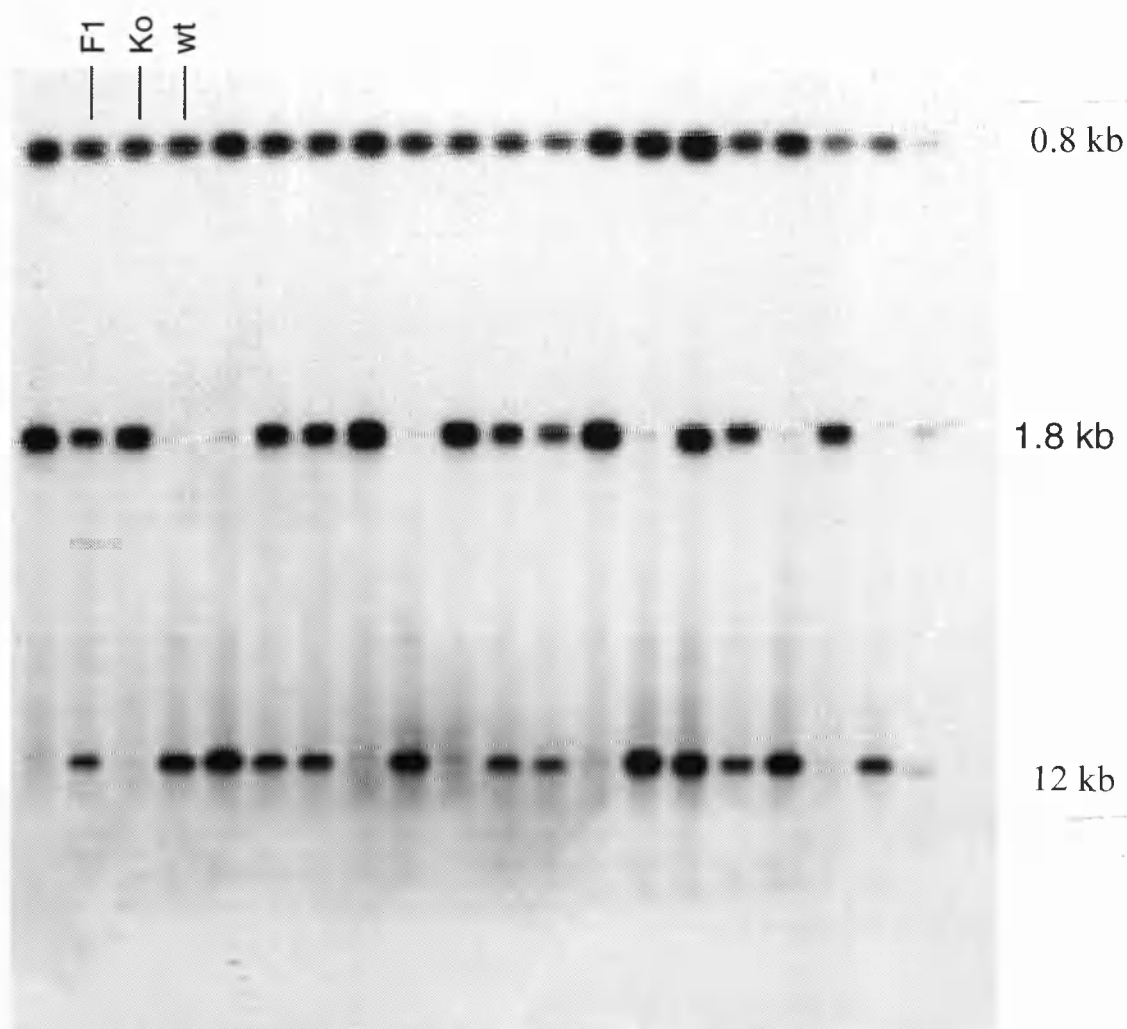
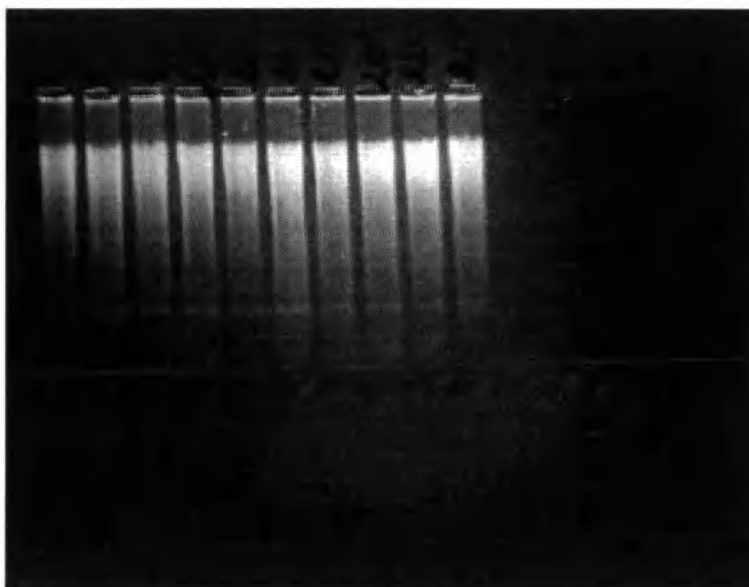
The Thy-1<sup>-/-</sup> mice were backcrossed to 129/Sv mice, and the F1 progeny interbred to obtain a second F2 recombinant population. Thy-1<sup>+/+</sup> and <sup>-/-</sup> littermates from this second F2 recombinant generation were then compared in a number of behavioural assays. Traits such as the distinctively altered rear motor co-ordination were rarely recovered, and were not linked to the Thy-1<sup>-/-</sup> population. Studies showed that no Thy-1 protein was detected in the Thy-1<sup>-/-</sup> animals using a variety of assays both with polyclonal and monoclonal antibodies.

Part of the results of this thesis were obtained with the material from the initial F2 recombinant generation, latter results with mice from the second recombinant generation obtained after backcrossing to 129/Sv mice. In both cases, Thy-1<sup>+/+</sup> and <sup>-/-</sup> littermates were compared.

To identify genotype, I used Southern blotting of DNA prepared from tail biopsy. Genomic DNA was purified and digested with *Bam* H1 (Fig 7). A southern blot of *Bam* H1-digested genomic DNA was then hybridised using a <sup>32</sup>P labelled *Apa*1 fragment of Thy-1 (Fig 8). The digest gave rise to bands that migrated to the expected positions and with intensities characteristic of a correctly targeted event. The positions of the bands were as follows: wild type (Thy-1<sup>+/+</sup>) band was seen at 12kb and 0.8kb; F1 (Thy-1<sup>+/-</sup>) at 12kb, 1.8kb and 0.8kb and knockout (Thy-1<sup>-/-</sup>) at 1.8kb and 0.8kb (Fig 8).

**FIG 7.** A gel image showing migration of *Bam* H1 digested DNA. The concentration, purity of DNA and efficiency of digestion was determined using a mini-gel. A uniform streak shows efficient digestion and the intensity of the streaks provide an estimate of the DNA concentration.

**FIG 8.** A Southern blot showing 0.8 kb 3' fragment with all Thy-1 forms that acts as a loading marker ( *top* band), a 12 kb form ( *bottom* band) with normal Thy-1 (+/+), half intensity in +/- mice), and a new 1.8kb band with the inactivated gene (middle band with -/-, half intensity in +/-).



**3.2. 1**      **IMPROVED METHOD FOR DRG CELL DISSOCIATION**

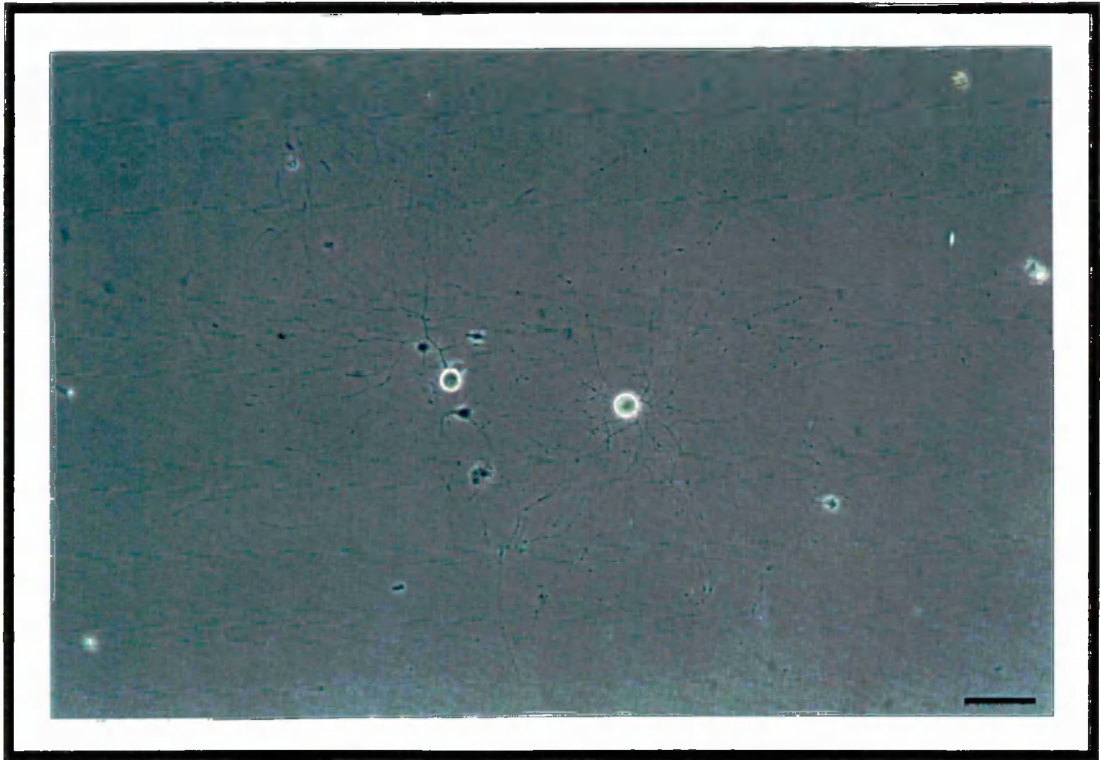
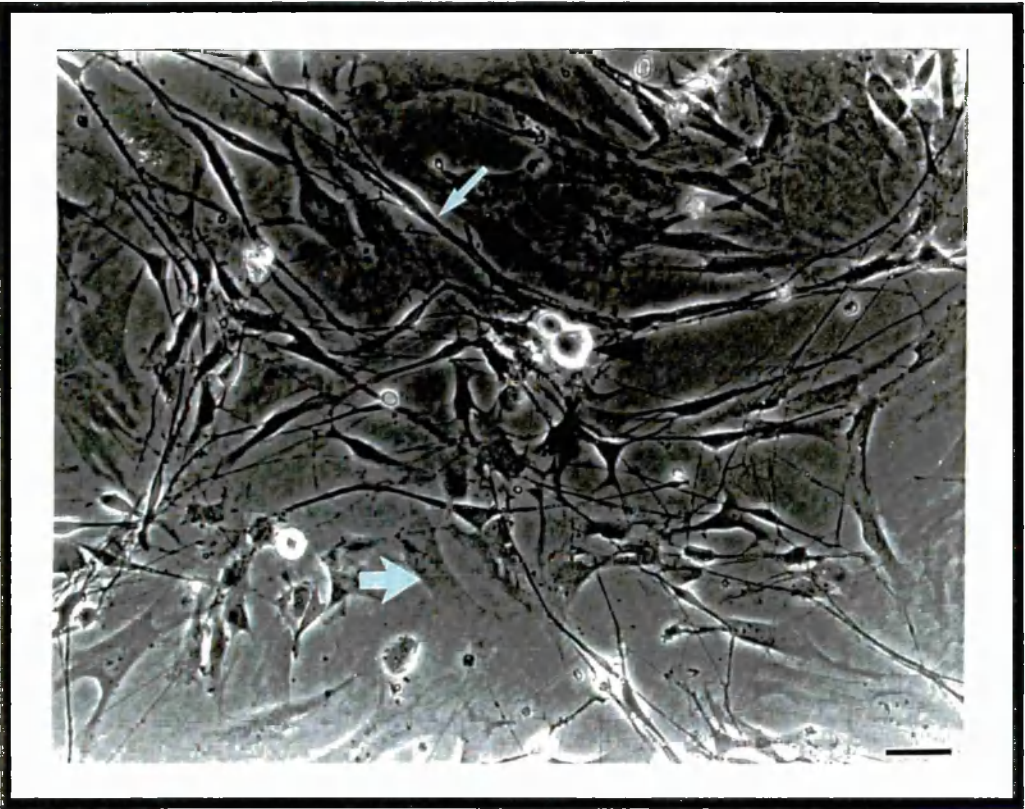
Lindsay's (1989) method for deriving dissociated DRG cells from adult animals was optimised. In order to measure neurite outgrowth of individual DRG cells on defined substrata, it was essential to obtain viable neurons that were growing as single isolated cells. While the dissociation procedure had to be mild enough to retain maximum viability and metabolic integrity of the cells, it had also to be vigorous enough to obtain dissociated neurons free of other contaminating cell types (fibroblasts, Schwann cells) (Fig 9). Fibroblasts were typically flat with clearly distinguishable lamellipodia and Schwann cells were identified by their spindle shaped morphology. Some Schwann cells persisted in our cultures and although they may not have directly affected the neurite growth over the 22 hour time period of these experiments, they may have contributed growth factors (Heumann *et al.*, 1987a) to the growth environment so as to indirectly influence the neurite growth (Yasuda *et al.*, 1990).

Plating density influenced the percentage of cells sending out neurites. At high cell plating density (1000cells/35mm dish) more DRG neurons sent out processes but the neurite outgrowth from adjacent cells overlapped thus preventing measurement of neurite length over most of the plate (Fig 10). It was found that a plating density of 300 cells/dish optimised the proportion of viable single neurons sending out neurites that did not overlap with their neighbours.

**FIG 9.** DRG cells (arrow head) plated on laminin. Fibroblasts (large arrow) and Schwann cells (small arrow) contaminate the cell culture if the dissociation procedure is not efficient. Bar 100  $\mu$ M.

**FIG 10.** DRG cells plated at a high cell density (1000 cells/35mm dish). DRG cell neurite outgrowth overlapping making accurate measurement of neurite outgrowth difficult. Bar, 100  $\mu$ M.

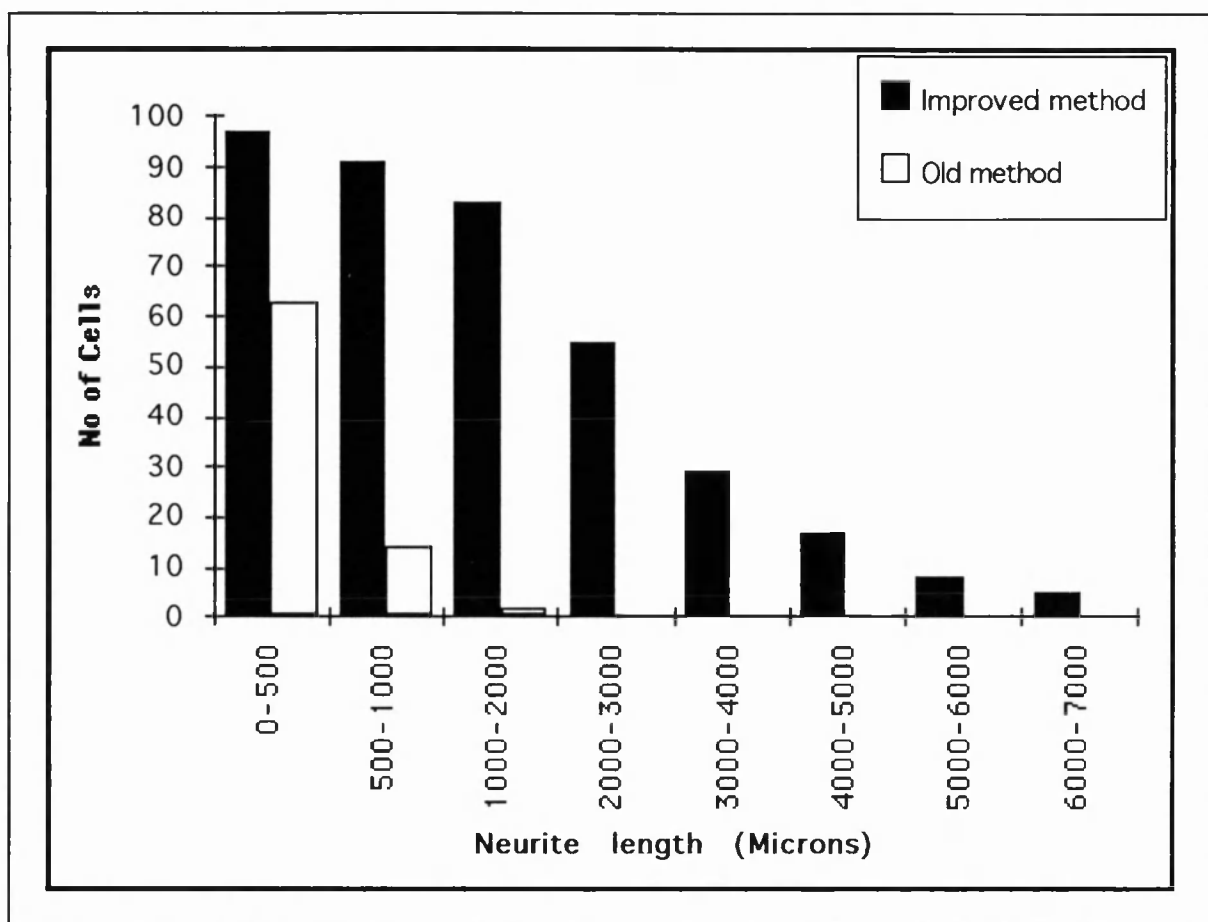




In early experiments, neurite outgrowth from adult DRG neurons on laminin was less exuberant and total neurite length did not exceed 5000 microns 24 hours after plating. By optimising the experimental conditions, I now routinely obtain a total neurite outgrowth of 7000 microns by the fastest growing DRG neurons on laminin at 18 hours (Fig 11). More cells are growing longer neurites and the neurites are more robust and remain intact and firmly attached to the substrate throughout the immunolabelling process. This improvement has been due primarily because with practice I have become much faster at getting ganglia into culture, and I have kept them prior to dissociation, in a drop of pH stable medium to optimise oxygenation. This contributed to maintaining viability of DRG cells. To decrease preparation time, I modified Lindsay's (1989) collagenase digestion (0.125% collagenase for 2 X 90 minutes) to be one 80 minute incubation in 250  $\mu$ l of 0.15% collagenase spread as a film over the ganglia. I found I could halve the trypsin concentration to 0.125%, and again I used minimal volume to maximise oxygenation.

Gentle trituration using an eppendorf pipette tip resulted in a cell suspension in which approximately 2% of the cells were in the form of a clump consisting of more than two cells. Further trituration dissociated the cell clumps but often the viability of the cells suffered. Examination of cell suspension showed that over trituration selectively effected the viability of the large diameter DRG cells compared to the medium to small DRG cells.

Like the adult DRG, dissociation time for embryonic DRG cells was kept to a minimum and manipulation was conducted in minimal volume of medium to achieve robust viable cells. The optimum time is a compromise between neuronal damage due to over-trypsinisation and damage due to vigorous trituration required to dissociate under-trypsinised tissue.



**FIG 11.** Graph showing neurite length at 18 hours in dissociated adult DRG neuron using two different protocols. The new improved protocol was quicker and resulted in robust neurite outgrowth. The longest neurite length using my improved method was more than 70% longer and more neurons grew longer neurites. At 18 hours, 2% of the cells were growing neurites longer than 2000 microns using the old method and more than 80% of the cells were still extending neurites longer than 2000 microns using the new protocol.

*In vivo* and *in vitro*, NGF has been shown to be necessary for survival of developing sensory neurons but not all mature sensory neurons (Johnson *et al.*, 1980; Barde *et al.*, 1980; Schwartz *et al.*, 1982; Lindsay, 1988a ). NGF was added to adult and embryonic DRG cultures to maintain constancy in the experiments.

### **3.2. 2**

### **CELL VIABILITY**

For neurite outgrowth assay, it was essential to obtain maximum numbers of viable, phase-bright, robust cells such as to be fully representative of the various known subpopulations (Scott, 1992) of primary sensory neurons. Viability of dissociated adult DRG cells was scored using fluorescein diacetate (FDA) test (Table 1) and confirmed using trypan blue dye exclusion test. Trypan blue stained dead cells blue and the viable live cells did not take up the dye and were phase bright (Fig 12 A : adult DRG neurons ; D : embryonic DRG neurons). In the FDA viability test, viable cells were labelled green and dead cells were granular and did not label with FDA (Fig 12 B and C : adult DRG neurons; E and F : embryonic DRG neurons). Percent viable cells was calculated by counting the total number of cells in several optical fields and total number of green FDA labelled cells in the same field. I was able to determine that my procedure yielded dissociated neurons that were more than 90% viable for embryonic (Fig 12 D,E and F- data not shown) and greater than 80% viable for adult neurons and of these, more than 60% of adult DRG neurons were sending out non-overlapping neurites after 24 hours in culture. Using FDA dye, mean viability was calculated at 87% for five adult DRG preparations and is shown in Table 1 below.

**Table 1.** Viability of dissociated adult DRG neurons using fluorescein diacetate.

Preparation No	Total No of cells	No of cells labelled with FDA	% age Viable cells
1	136	108	80
2	144	110	77
3	210	184	88
4	133	110	83
5	110	85	77

### **3.2.3**                      **MORPHOLOGY AND IMMUNOCYTOCHEMISTRY**

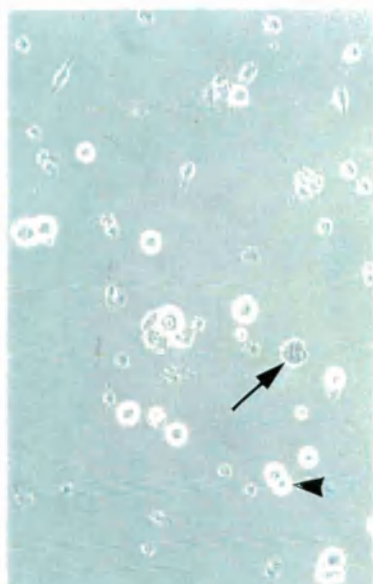
#### **3.2.3-1**                      **DRG CELLS**

After dissociation of adult DRG, I observed a heterogeneous population of phase-bright, spherical cells with perikaryonal diameter ranging from 15-70 microns (Fig 13A,B and C). Highly granular cells were not in good health and often died after 4-6 hours in culture. Embryonic DRGs were uniform and cell diameter was 15-30 microns (Fig 14).

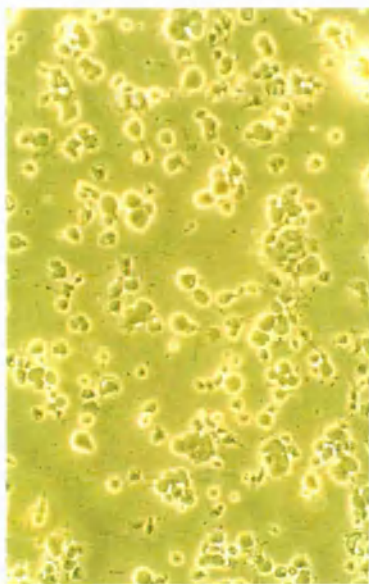
The RT97 monoclonal antibody was used to distinguish between two cell types (Fig 15A) in dissociated DRG cultures, since small dark (SD) cells (small arrow) are NF poor and L (large) cells (arrow head) express large quantities of phosphorylated form of the 200kDa neurofilament subunit . Unlike the adult, the embryonic DRG cells were labelled to the same intensity (Fig 15B). Not all large cells (greater than 35  $\mu$ M) were uniformly stained with RT97. The pattern of intensity varied considerably. Some cells were heavily stained and and were packed full of neurofilaments. Others were more lightly stained often with a diffuse pattern.

**FIG 12.** Viability test using trypan blue dye exclusion test for adult (A) and embryonic (D) DRG cell preparation. Dead trypan blue positive cells (arrow) and phase-bright (arrow head) live cells are seen. Fluorescein diacetate (FDA) stain for adult (phase: B and FDA: C) and embryonic (phase:E and FDA :F) DRG neurons. Live cells are labelled green. Bar 100  $\mu$ M.

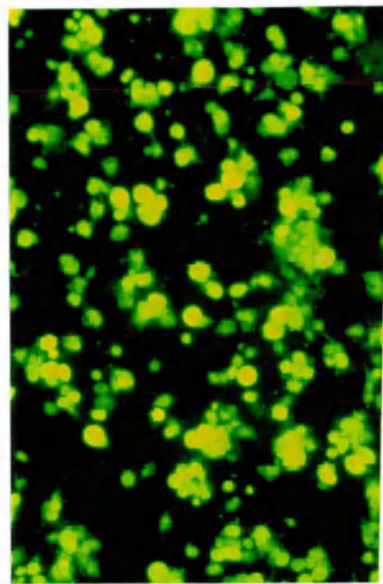




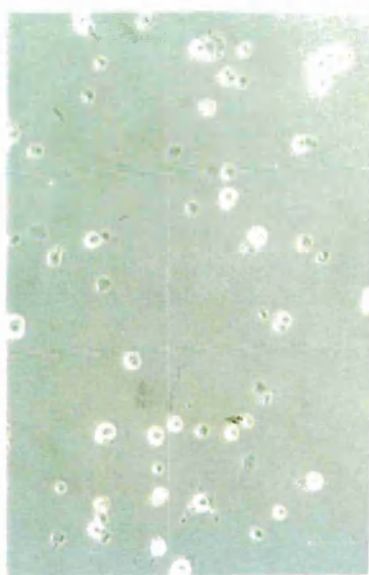
**A**



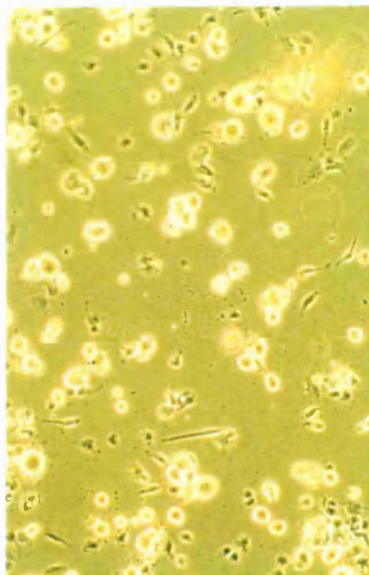
**B**



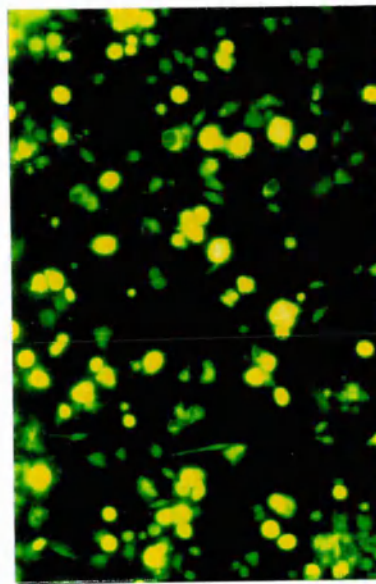
**C**



**D**



**E**



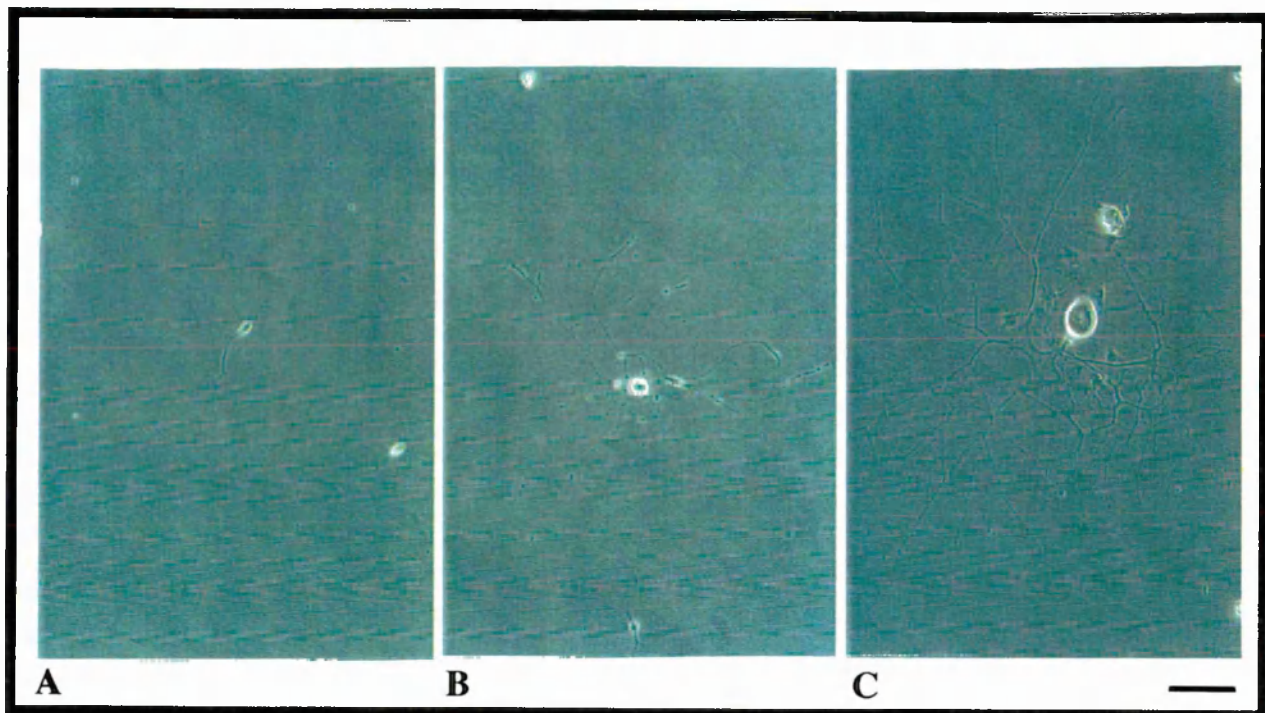
**F**



**FIG 13.** Phase picture of heterogenous population of adult DRG cells. A : a small, B: medium and C : large diameter DRG cells on laminin. Bar 100  $\mu$ M.

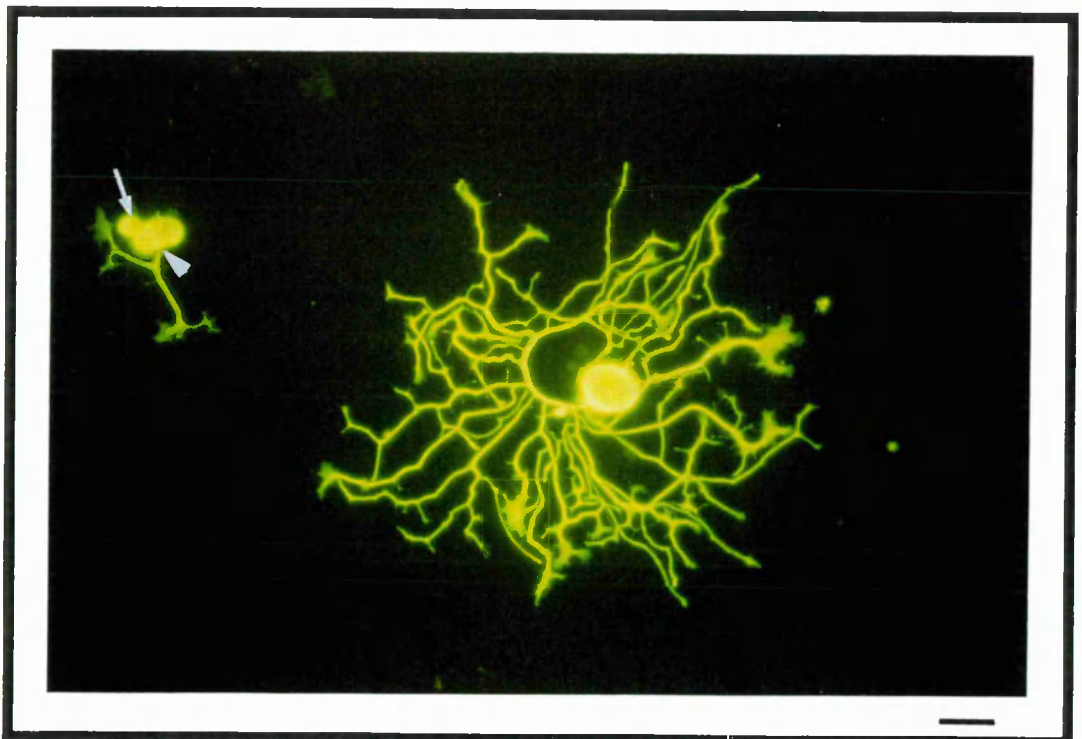
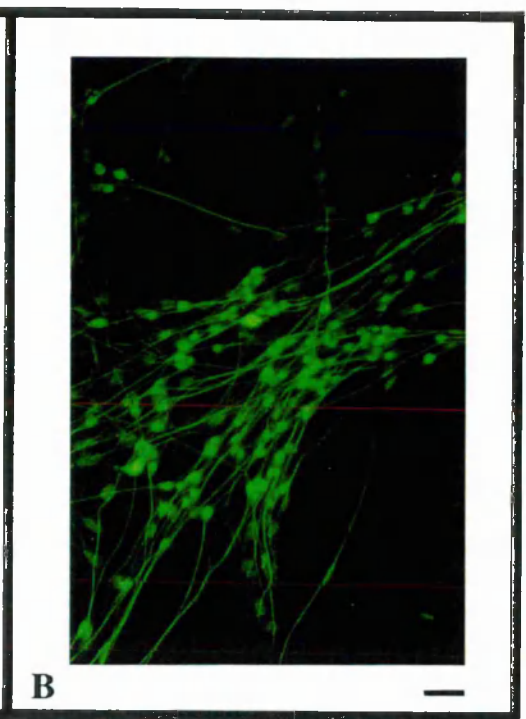
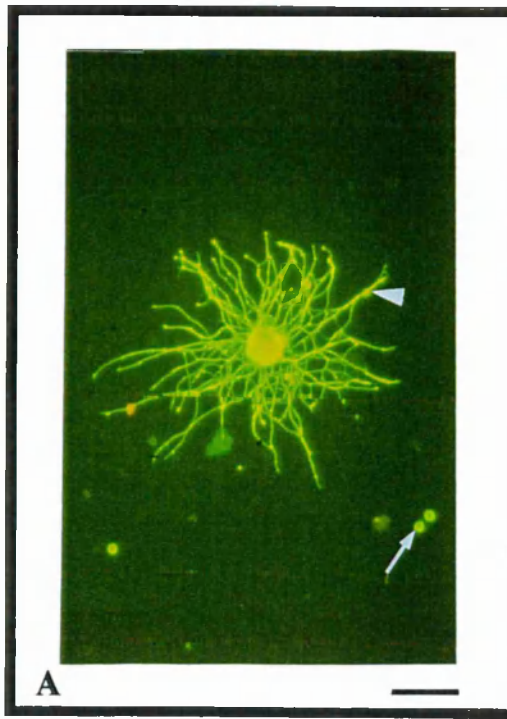
**FIG 14.** A phase picture showing a homogeneous population of embryonic DRG cells. Bar 100  $\mu$ M.





**FIG 15.** Adult (A) and embryonic (B) dissociated DRG neuron population labelled with anti-neurofilament antibody RT97. A: Intensely labelled large diameter cells (arrow head) and weakly labelled small diameter (arrow) DRG cells. B: All embryonic cell population label with equal intensity. Bar 100  $\mu$ M

**FIG 16.** Immunofluorescent photomicrograph of heterogeneous population of DRG neurons grown for 18 hours on laminin and labelled with a cocktail of RT97 and TuJ 1 antibodies. Adult DRG neurons showing a large cell (perikaryon diameter of 58 microns) and to the left a small cluster of cells (arrow shows a small cell of 20 microns cell diameter). Satellite cells are attached to the cell body (small arrow). Bar 100  $\mu$ M



To label embryonic as well as the large and the small diameter adult neurons, a cocktail of RT 97 and anti- $\beta$ -tubulin antibody (Fig 16) was used. Unlike embryonic neurons, 10-14 % of adult neurons had 1-5 satellite (arrow) cells associated with their cell bodies. Within the time course of these experiments, these cells remained attached to the cell body and did not migrate onto the rapidly growing neurites.

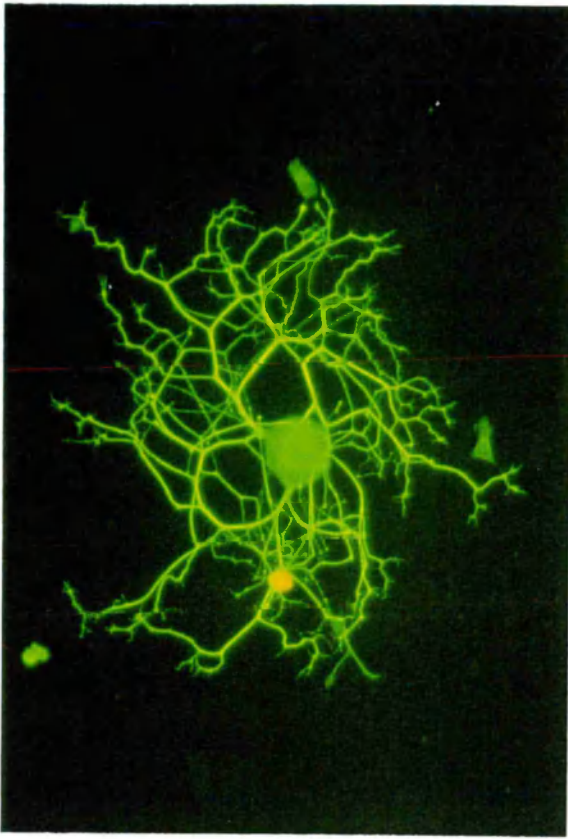
After dissociation, the DRG cells were spun through a column of 12% BSA. This procedure removed the cell and myelin debris and non-neuronal cells. The cells were then plated onto the substrates.

By 10 hours on laminin, the medium to large sized neurons (30-70 microns) extend elaborate, highly branched neurites that radiate out from the cell body (Fig 17A). The neuronal growth cones recognise laminin as a growth permissive environment the neuritic growth on this substrate is profuse and highly arborised for the medium and large neurons. Small diameter DRG (15-30 microns) extend neurites which are of a simpler morphology and usually bipolar (Fig 17B). The more homogeneous population of embryonic DRG cells extended fine bipolar or unipolar linear neurites from the cell body (Fig 18).

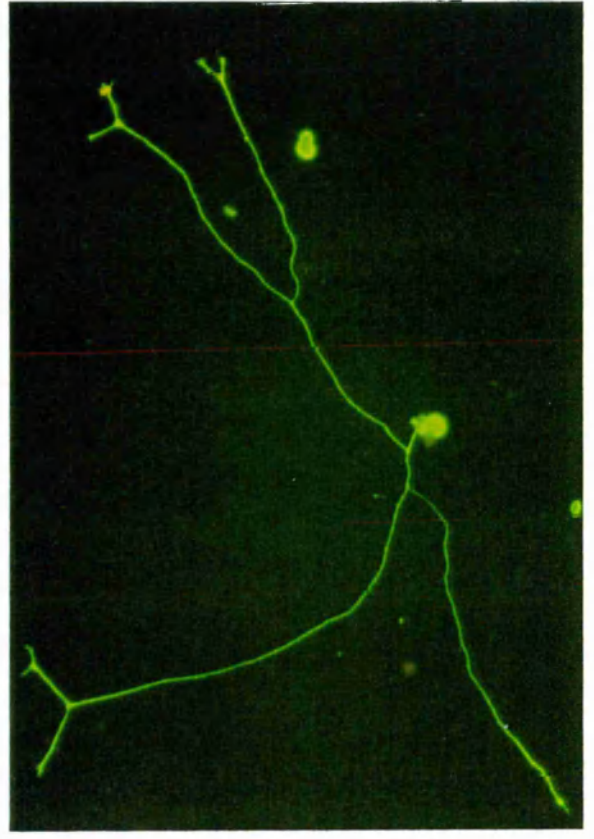
**FIG 17.** Adult DRG neurite growth pattern on laminin after 10 hours in culture. Large diameter (30-70 microns) DRG cells (A) extend highly branched, arborised neurites and small diameter DRGs (B) extend less complex bipolar neurites. Bar 100  $\mu$ M

**FIG 18.** Embryonic DRG cell neurites are simple bipolar. Bar 50  $\mu$ M

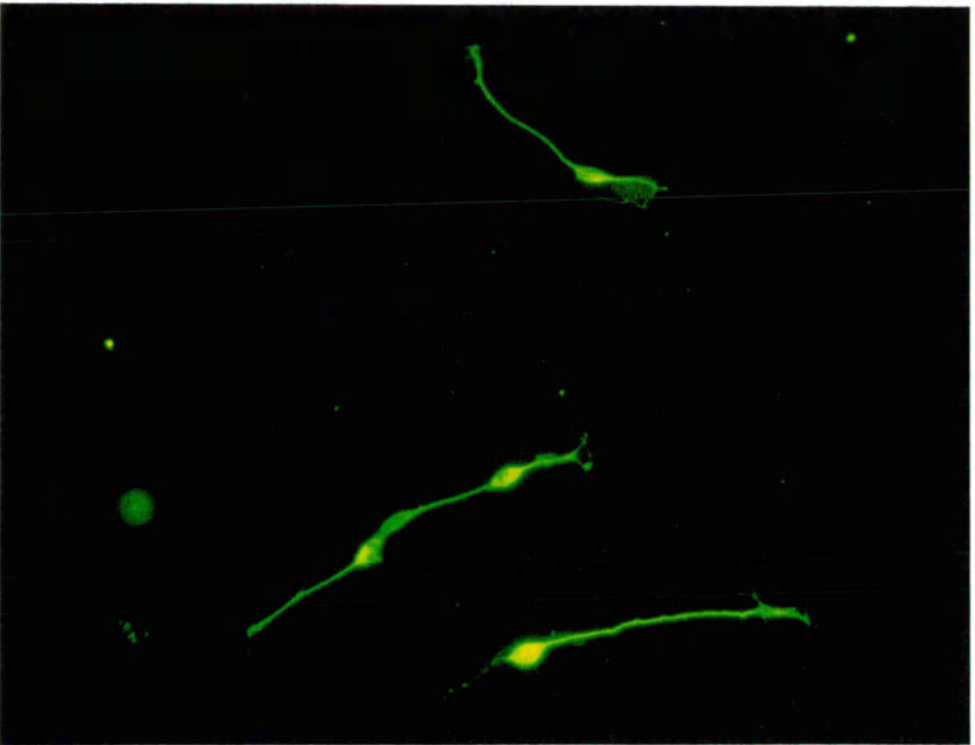




A



B



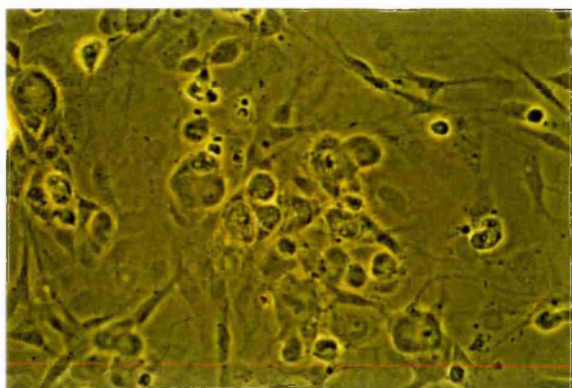
To assess the expression of two peptides in adult (Fig 19) and embryonic (results not shown here) cultured mouse DRG cells, the cells were immunolabelled after 18 hr in culture with antibodies to CGRP and substance P which are known to be expressed on subsets of adult rat DRG neurons (Willis and Coggeshall, 1992). Neither antibody stained the occasional Schwann cell or fibroblast present in culture. Substance P (Fig 19 A,B) immunoreactivity was observed in more than 70% of all adult DRG neurons labelling cells belonging to all three size classes. Neuronal cell bodies were intensely labelled and I observed punctate labelling of the neurites, indicating vesicles carrying peptides. Of the population of unlabelled cells, the large diameter neurons formed the largest group sometimes exhibiting weak, diffuse autofluorescence. Embryonic DRG neuron labelling was diffuse and uniform, I concluded therefore that this was non-specific labelling. When adult DRG neurons in culture were labelled with antibody to CGRP (Fig 19 C,D) strong immunoreactivity on both cell bodies and neuronal processes was seen in neurons belonging to all three size classes. More than 80% of embryonic DRG cells did not express CGRP and of those that did show immunoreactivity, the neurites labelled less intensely than adult DRG. Modulation of expression of CGRP and substance P due to the addition of NGF has been shown but the effect is only seen after the cells have been in culture for 48 hours (Lindsay *et al.*, 1989). Since DRG cultures for these experiments were fixed at 22 hours, we are able to rule out the role of NGF in altering expression of CGRP and Sub P. Expression of CGRP and substance P in Thy-1 Ko animals was similar to the Wt animals.

### 3.2.3-2      ASTROCYTES

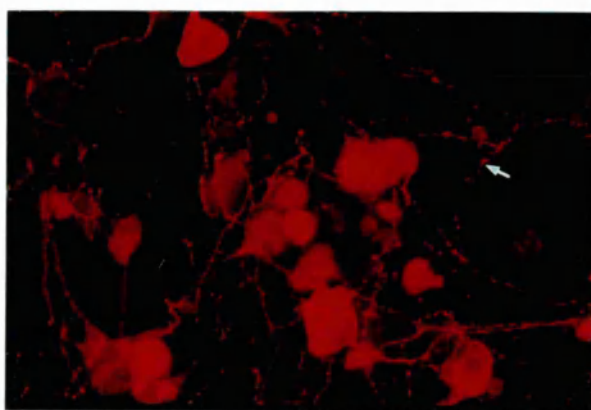
Mature astrocytes derived from *corpus collosum* of neonatal rat showed large, flat, fibroblast-like, non-process bearing cell body (Fig 20A) and were identified by specific antibody against glial fibrillary acidic protein (GFAP) (Fig 20 A,B). Mature astrocytes were immunopositive for Thy-1 (Fig 20 C,D). The labelling for Thy-1 was

**FIG 19.** Neuropeptide expression in adult (A-D) DRG cells. Cells plated on laminin were fixed after 12 hours and labelled for Substance P (A:phase, B:TRITC labelled) and CGRP (C:phase, D:FITC labelled). Bar 100  $\mu$ M.

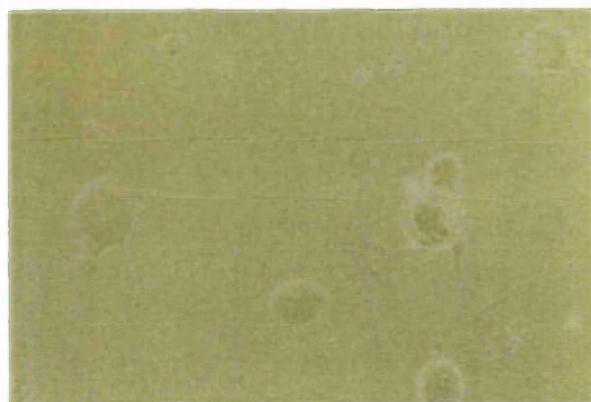




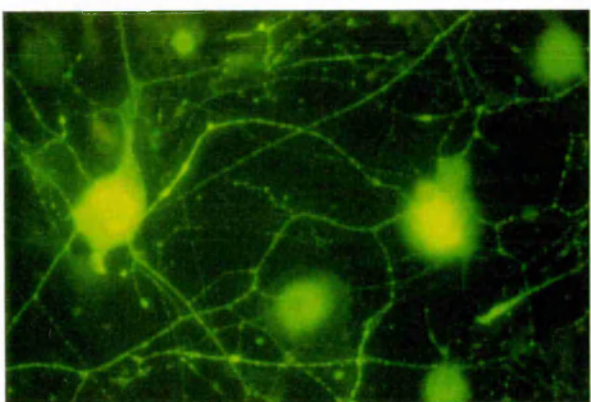
A



B



C



D

granular and more intense at the cell boundary. Astrocyte cultures were more than 97% pure and fibronectin expression was negligible (Fig 20 E,F). Chondroitin sulphate proteoglycan immunocytochemistry (Fig 20 G,H) showed occasional streaky patches of granular labelling-generally near the periphery of astrocytes.

### **3.3**                    **AXONAL GROWTH STUDIES**

#### **3.3.1**                    **GROWTH OF ADULT AND EMBRYONIC DRG NEURONS ON LAMININ AND ASTROCYTES**

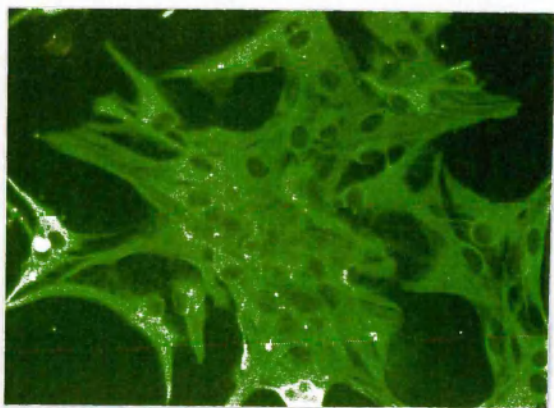
Neurite growth profile of adult and embryonic wildtype DRG on laminin and astrocytes at 5, 18 and 24 hours was constructed by tracing neurites on photographs taken under fluorescence optics and is shown in Fig 21.

Under optimal conditions on laminin, more than 80% of DRGs adhere within 3 hours of plating. It was observed that more than 40% of adult neurons begin to extend arborised neurites by 6 hours in culture and in excess of 60% of embryonic DRGs were extending bipolar neurites after 6 hours in culture. I observed that 78-92% large and small perikaryon adult DRGs adhered to the astrocytic substrate within 2 hours of plating and this included a small population (<2%) of non-viable DRGs. By 6 hours, 15-30% of adult neurons were extending stubby neurites on astrocytes and on laminin a network of neurites was evident. More than 50% of embryonic DRGs were sending long fine neurites by 6 hours.

**FIG 20.** Mature astrocytes immunolabelled astrocytes were positive for GFAP (A: phase; B: GFAP.,FITC labelled) and Thy-1 (C: phase; D: Thy-1.,TRITC labelled) and predominantly negative for fibronectin (E:DAPI; F: fibronectin., FITC labelled), chondroitin sulphate proteoglycan (G: DAPI; H: CSPG., TRITC labelled). Bar 100  $\mu$ M.



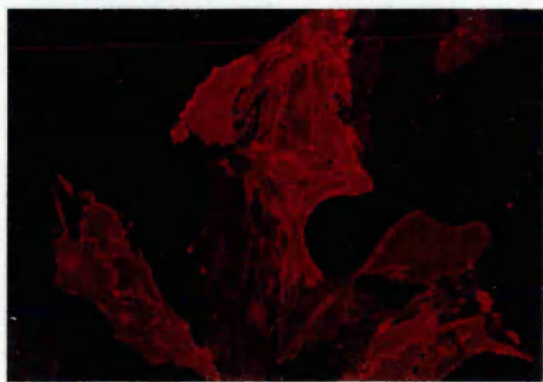
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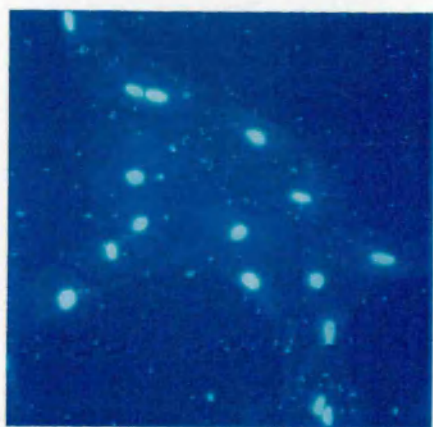
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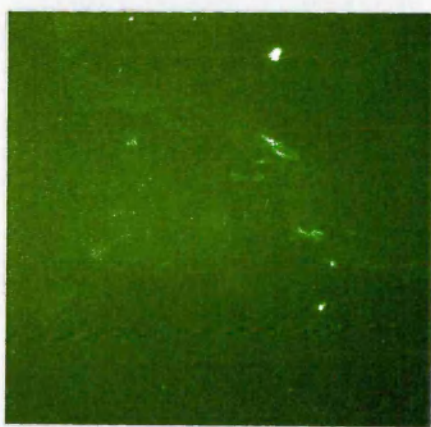
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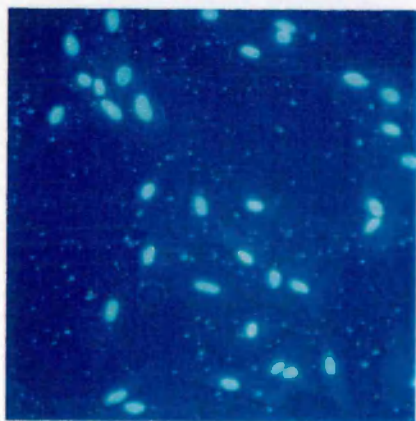
D



E



F



G



H

On laminin, the improved dissociation procedure yielded vigorous neurite outgrowth from both embryonic (E12) and adult (P28) neurons. At 5 hours on laminin, the embryonic DRG cells were extending simple, fine unipolar or bipolar neurites (Fig 21), a pattern that was largely retained at 16 hours and at 22 hours with increasingly longer neurites. DRG neurons derived from P 28 (adult) mice were projecting thick neurites with prominent growth cones at 5 hours and by 16 hours in culture the cells had established a morphology that ranged from simple pseudo-unipolar to complex multipolar patterns with extensive secondary branching. At 22 hours a complex pattern of arborisation was seen.

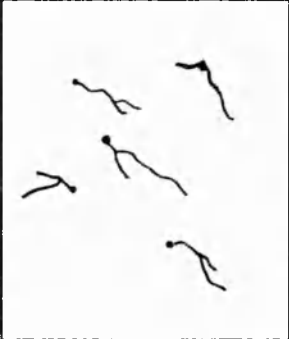








The overall pattern of neurite outgrowth on astrocytes was similar to that on laminin. Embryonic DRG neuron growth was similar on laminin and astrocytes but growth of adult DRG neurons was retarded when compared to laminin. Growth of embryonic and adult DRG neurons on astrocytes was monitored. DRG neurons (300 DRG cells/35mm dish) were plated on a confluent, flattened monolayer of mature astrocytes. More embryonic DRG neurons extended neurites at 5 hours on astrocytes compared to adult (less than 30% of total) neurons. At 5 hours, the embryonic neurones extended simple neurites (Fig 21). After 16 hours in culture the embryonic DRG cells had grown long neurites which were branching. By 22 hours, DRG neurites from embryonic cells had grown long extensions which often branched at the leading edge.

The morphology of adult DRG neurite outgrowth over astrocytes suggested this was a less favourable substrate than it was for the embryonic neurons. The adult DRG neurites tended to form a network (Fig 21) on the DRG cell body a feature which was largely absent on laminin, a more hesitant growth was observed away from the cell body. Branching was less arborised, fasciculation was observed and growth cones were not as prominent as on

laminin. Small diameter adult DRGs extended fine unfasciculated neurites on astrocytes. Adult DRG neurons grew shorter neurites than embryonic DRG neurons at 5 hours. Adult neurites at 5 hours were sometimes short, thick straight and unipolar, but the substantial majority were multipolar. Complex branching and anastomosing network of fine neurites that form fascicles were clearly evident at 16 hours. Often prominent growth cones were seen at the leading edge of the neurite outgrowth. Tangled neurites were visible around the cell body at 22 hours; away from the cell body, long profusely branched neurites were seen which fasciculated at intervals along their length. Adult neurites often formed loose fascicles of 2-8 fine processes which I was able to resolve and measure by using higher magnification, varying the focus and enhancing the video image. A sub-population of adult DRG neurons (about 8-12% of total cell population) which had smaller cell bodies (15-30 microns) extended fine long unbranched processes extending more than 3000 microns after 22 hours in culture. At 24 hours arborisation and fasciculation of neurites from some cells on laminin (Fig 22A) and astrocytes (Fig 22B) was too complex and intricate as to preclude accurate measurement of neurite outgrowth.

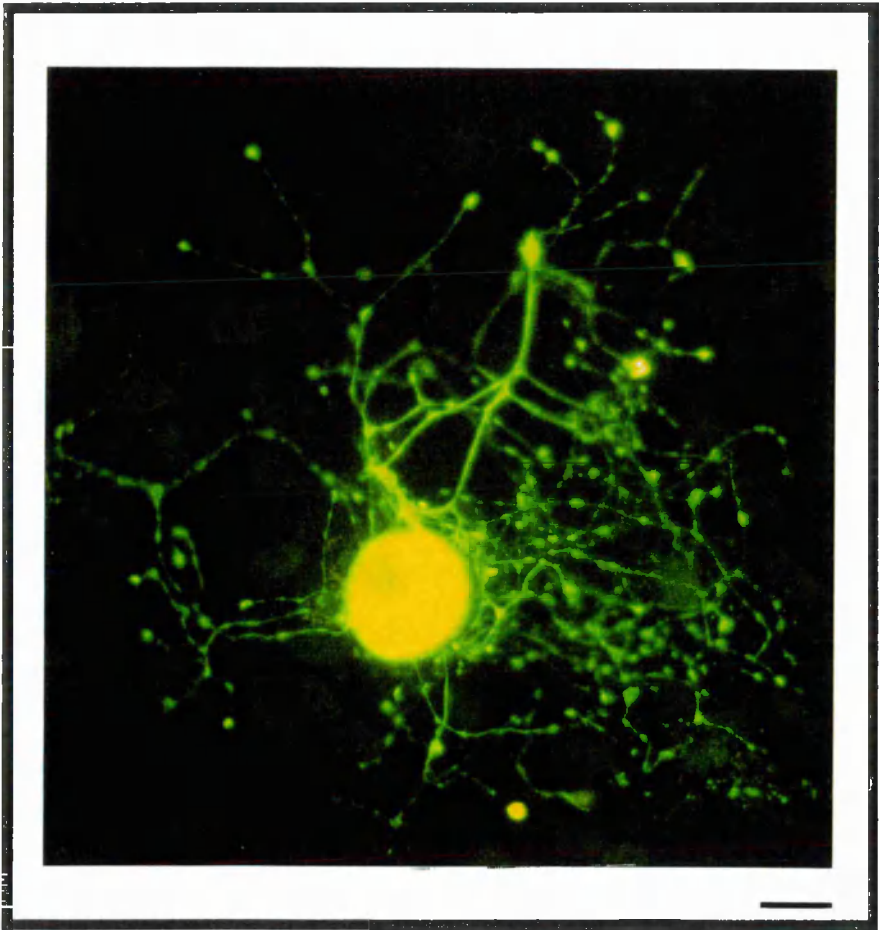
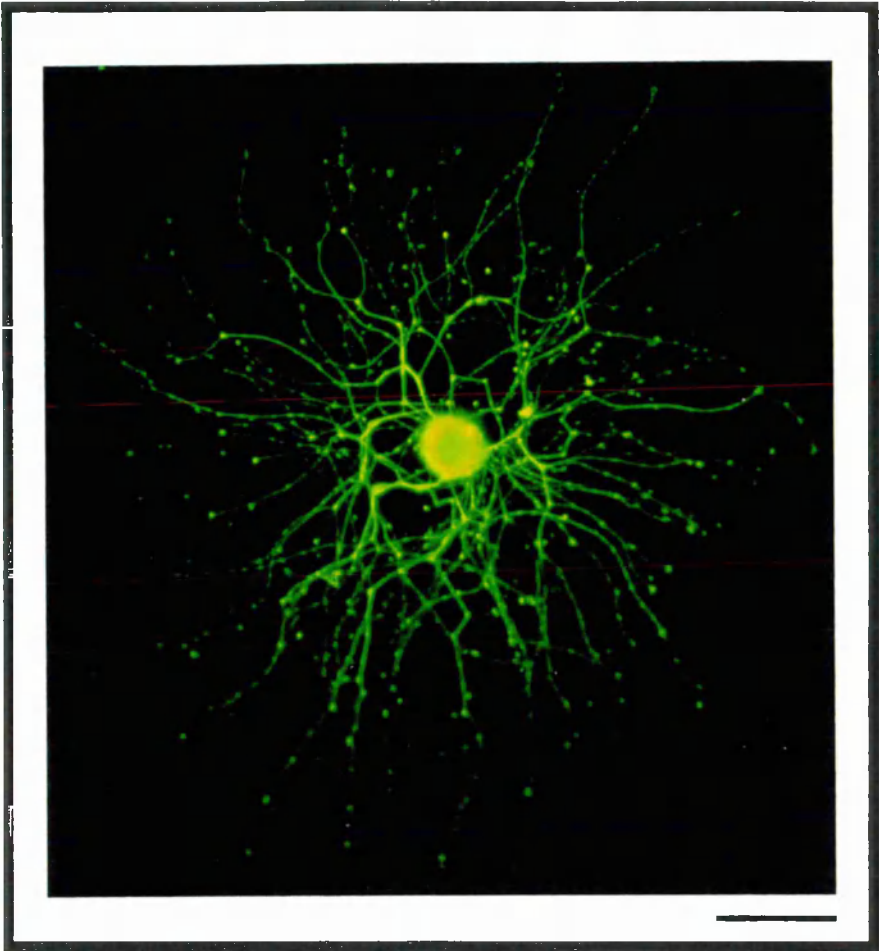
Thus the growth of neurites from embryonic DRG neuron differs in both its pattern and time of onset of neurite extension from that of adult DRG neurons. Growth of adult DRG on astrocytes at the same time point is less prolific and arborisation of neuritic growth more restricted when compared to the growth on laminin.

**FIG 21.** Profile of embryonic (left) and adult (right) DRG neurons grown for times indicated on laminin or astrocytes, fixed, labelled with RT 97 and TuJ 1, photographed under fluorescence optics and images were traced from photographs.

Embryonic		Adult	
Laminin	Astrocytes	Laminin	Astrocytes
5h			
18h			
22h			



**FIG 22.** Growth of DRG neurons on laminin (A. Bar 100 $\mu$ M) and astrocytes (B. Bar 30 $\mu$ M). A complex network of neurites is seen at 24 hours.



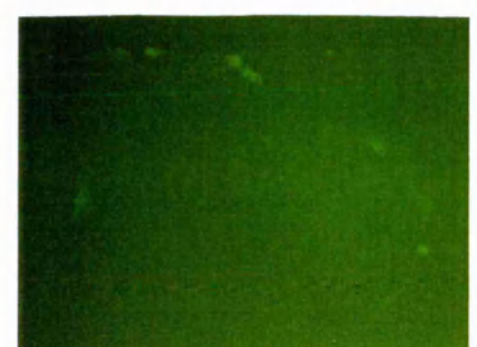
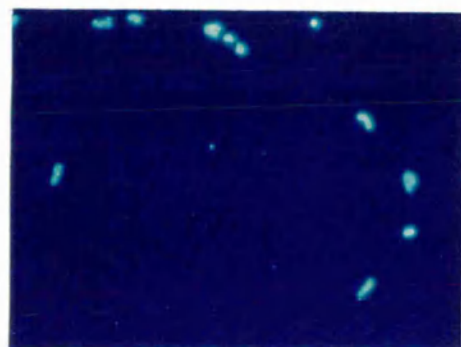
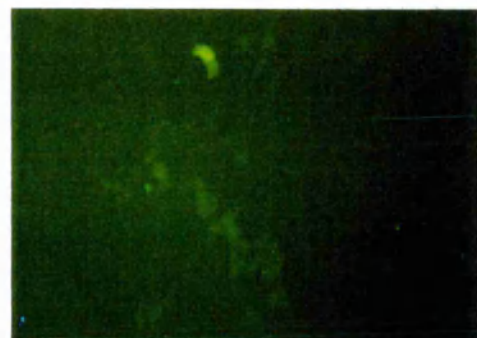
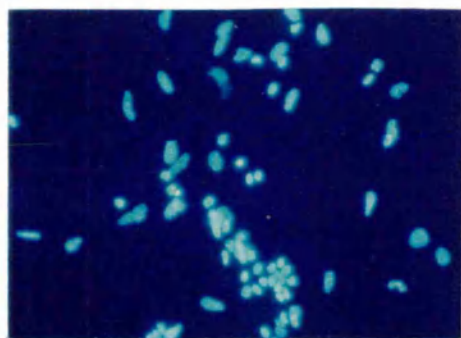
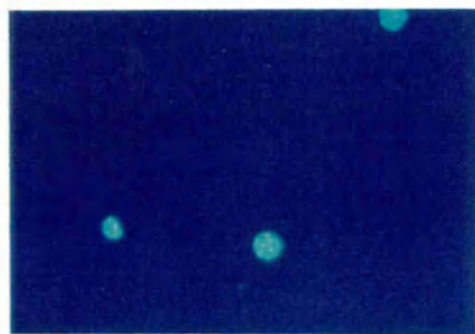
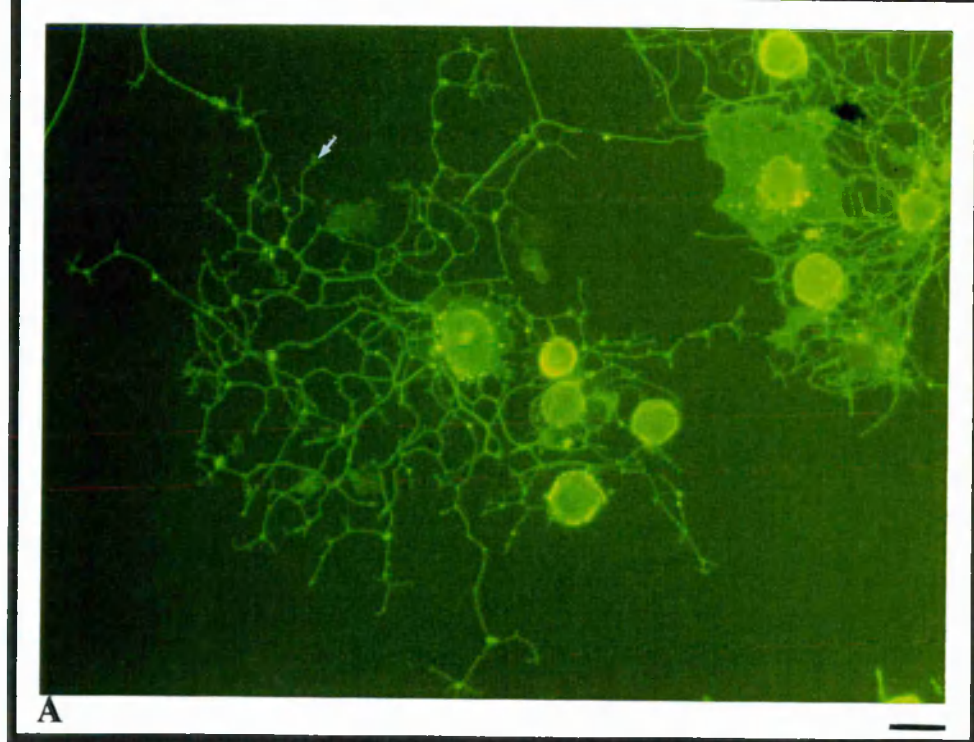
### **3.3.2 THE EFFECT OF THY-1 ON NEURITE OUTGROWTH**

#### **FROM ADULT DRG NEURONS ON ASTROCYTES**

In order to assess the effect of Thy-1 on axonal growth over astrocytes, the growth of neurites by DRG neurons from adult Thy-1 positive control mice (aged 27-30 days) of the 129/Sv strain (these will be referred to as Wt in the text) and from age-matched Thy-1 knockout transgenic mice bred from those obtained from the first F2 recombinant generation (these will be referred to as Ko in the text) was compared. It was necessary to confirm the *in vitro* Thy-1 status of DRG neurons derived from Wt and Ko adult and embryonic mice. Immunolabelling using rat 30H12 anti-Thy-1.2 antibody showed that all adult Wt DRG neurons were positively labelled for Thy-1 (Fig 23 A). Wt embryonic neurons, and both embryonic and adult Ko DRG neurons did not show any immunoreactivity for Thy-1 (Fig 23 B-G). Neurons were plated onto a preformed monolayer of astrocytes, or onto laminin as a control substrate. Culture dishes were removed for fixation and immunolabelled, normally at two or three time points within 17-22 hours after plating. The total neurite length was then measured. After 16 hours in culture, all neurons were firmly adherent and neurons were not washed off during fixation allowing accurate representation of all DRG populations for determination of neurite outgrowth.

In many other studies, the behaviour of individual growth cone (followed by time-lapse photography) or the length of the longest neurite measured from the cell body has been used as a parameter to follow the effect of substrate on neurite outgrowth. Given the complex morphology of most adult DRG neuronal outgrowth and the variation in morphology, with some cells with many multibranched but relatively short neurites, others with fewer but longer processes, and a minor population with very long unbranched neurites, it seemed that the only genuine comparison would be to measure total neurite length of each cell.

**FIG 23.** DRG neurons were labelled with anti-Thy-1 antibody and a FITC secondary antibody was used to visualise labelling. Adult Wt DRG cells were Thy-1 positive (A) Bar 50 $\mu$ M. Cell bodies, neurites and growth cones expressed Thy-1 (Arrows). DRGs derived from Thy-1 Ko adult were not labelled for Thy-1 (B: DAPI, C: FITC labelled. Bar 50 $\mu$ M). Both Ko (D:DAPI, E: FITC) and Wt (F: DAPI, G: FITC) embryonic DRGs were negative for Thy-1. D,E,F and G. Bar 100 $\mu$ M.



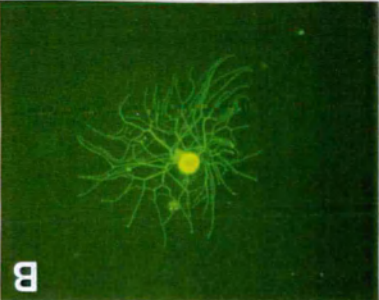
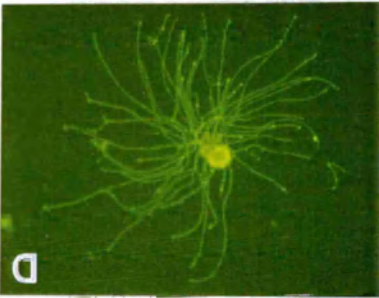
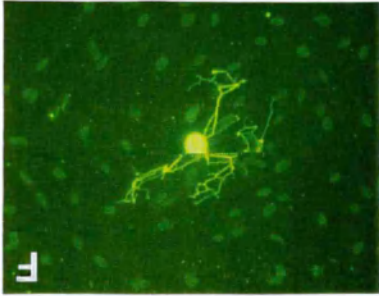
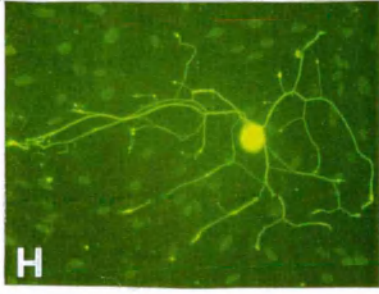
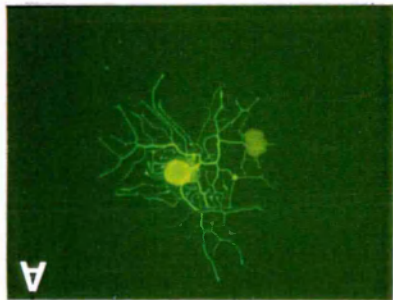
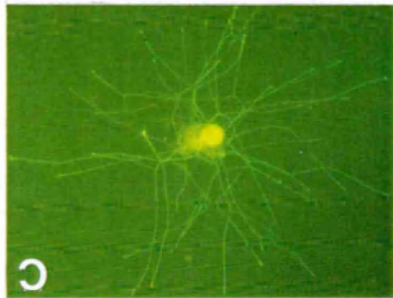
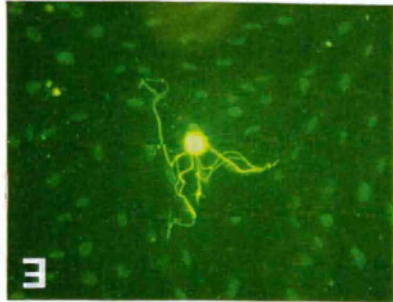
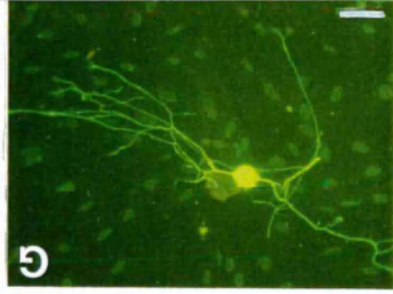
Wt (Thy-1 positive) (Fig 24, left panel) and Ko (Thy-1 negative) (Fig 24, right panel) DRG neurons were grown on laminin (Fig 24 A,B,C,D) and astrocytes (Fig 24 E,F,G,H). On laminin, Wt and Ko DRG cells extended multipolar, radial highly branched neurites at 18 and 22 hours (Fig 24 A-D). On a monolayer of astrocytes (Fig 24 E-H), a morphological difference became apparent at longer time points (22 and 24 hours). The tendency for neurites to fasciculate was less pronounced for the Ko cells. Compare Fig 24 E,G (Wt) with Fig 24 F,H (Ko). Moreover, it appeared that Ko DRG neurons extended processes which were longer than those from the Wt DRG neurons.

Neurite outgrowth from DRG neurons fixed at different time-points was measured using FluoVision Image analysis software. The results of a representative experiment are shown in Fig 25. In this experiment Ko DRG neurons grew marginally longer neurites on laminin than Wt DRG neurons (A: 17 hour, B: 22 hour). At shorter time points, i.e. less than 18 hours, Wt neurons often grew longer neurites than Ko cells (not shown). On astrocytes, in this experiment any difference in neurite length between the two types of neurons was marginal at 17 hour (Fig. 25C) but by 22 hour (Fig.25D) the Ko neurites had started to grow longer (on average, in this experiment 27%) than Wt DRG neurites.

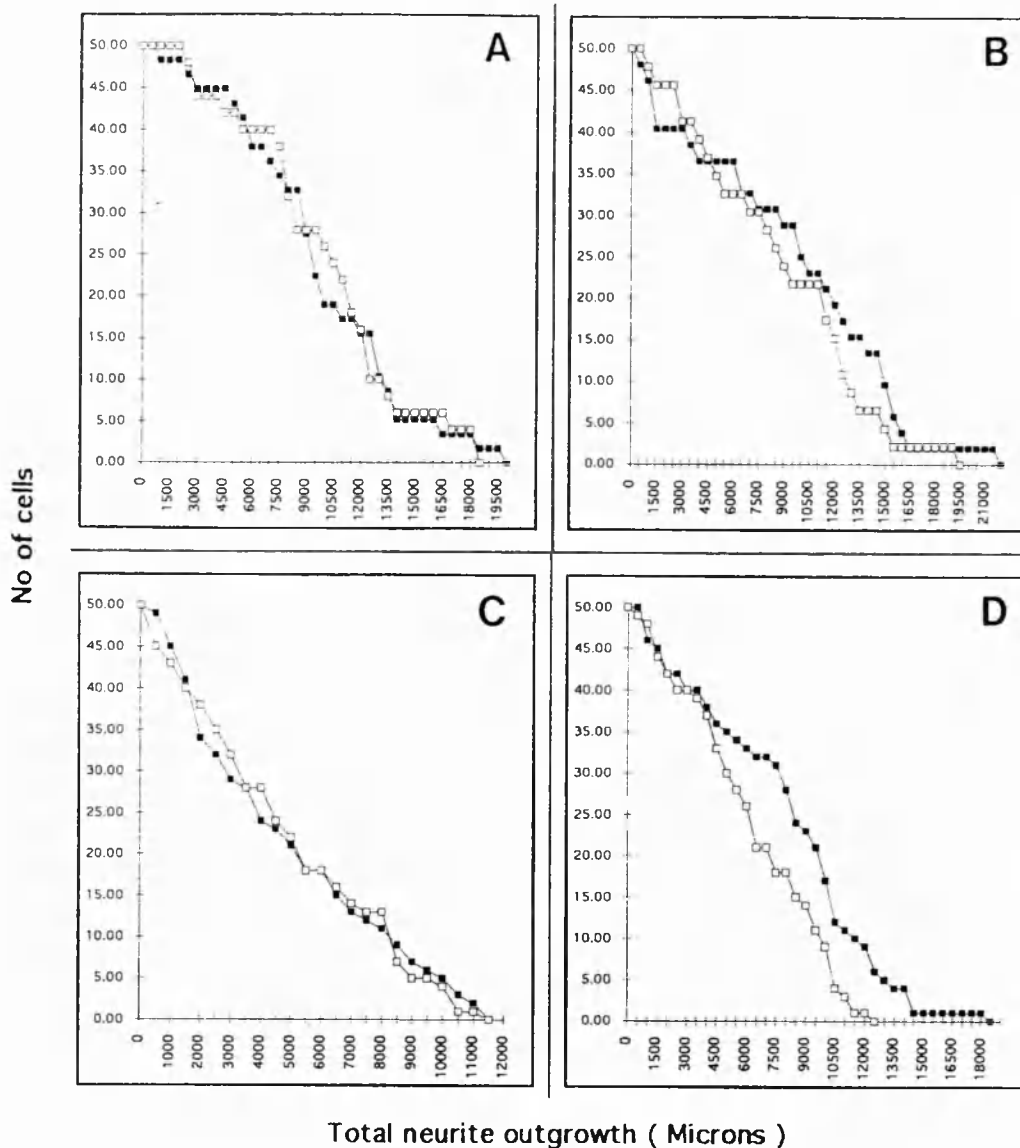
To test the significance of the difference in length of neurite outgrowth, the results of all experiments on laminin (7) were pooled, as were all the experiments on astrocytes (9). The average neurite length at all time points (17-22 hours) was calculated. The result of pooled data for growth on laminin and astrocytes is plotted in Fig 26. On laminin 391 Ko and 183 Wt neurons, and for growth on astrocytes a total of 483 Ko and 414 Wt neurons were measured and analysed. On a monolayer of astrocytes the difference was pronounced: Ko neurons on the average grew 10% further than Wt DRG neurons and this result was highly significant ( $p = 0.0001$ ). On laminin Ko neurites grew longer processes than Wt but the difference was not significant ( $p = 0.258$ ) due to problems measuring outgrowth by cells on laminin (see below).

**FIG 24.** Adult (P28) Wildtype (Wt; Thy-1 positive) (left panel) and Knockout (Ko;Thy-1 negative) (right panel) DRG neurons on laminin (A-D) and astrocytes (E-H) at 18 hr (A,B,E,F) and 22 hr (C,D,G,H). There was no difference in neuronal morphology between Wt DRG neurons (A,C) and Ko DRG neurons (B,D) at 18 and 22 hours. On a monolayer of astrocytes, Wt (G) DRG neurites fasciculated more than Ko DRG neurites (H). Bar, 50  $\mu$ M.

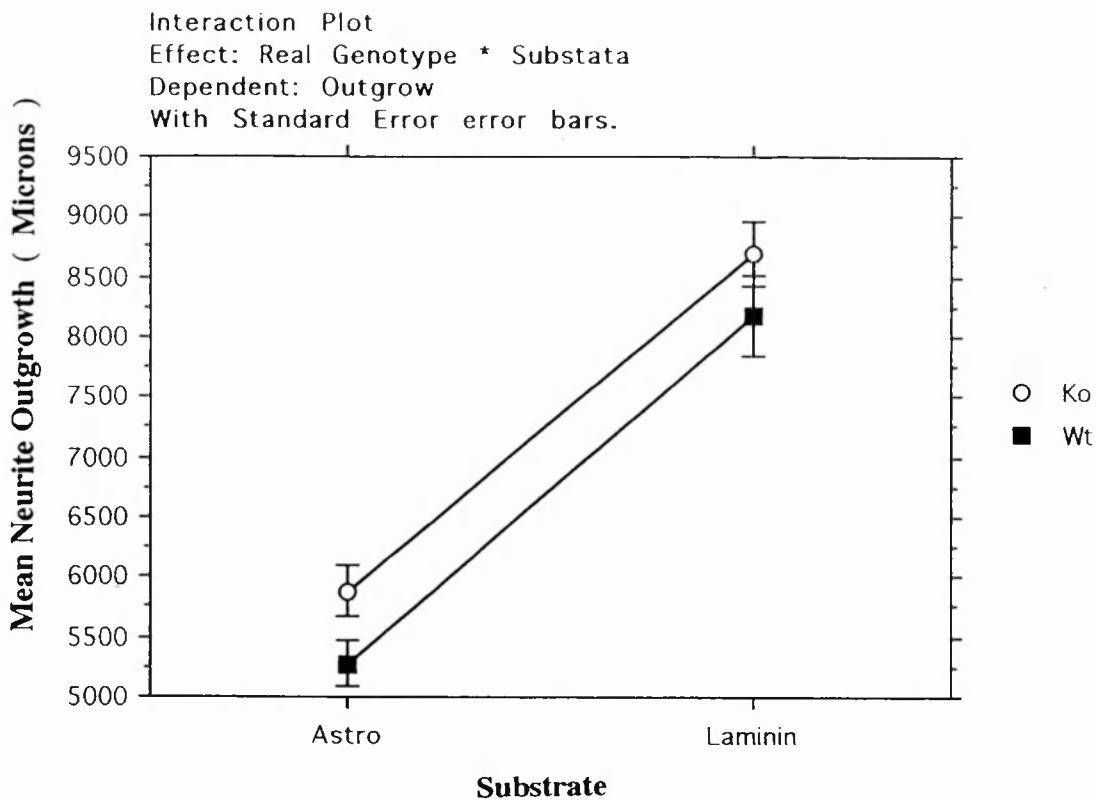








**FIG 25.** Neurite outgrowth from adult (P28) Thy-1 positive (□) (Wt) and Thy-1 negative (■) (Ko) cells on laminin at 17 and 22 hour (A,B) and a monolayer of astrocytes at 17 and 20 hour (C,D). Neurite outgrowth of immunofluorescently labelled cells was measured in eight different sectors of a 35 mm dish using a fluorescence microscope linked to an image analysis system. Results are from a single experiment.



**FIG 26.** Thy-1 positive (WT) and Thy-1 negative (Ko) adult (P28) DRG cell neurite outgrowth on a monolayer of astrocytes and laminin. Total of 483 Ko and 414 Wt DRG neurons on astrocytes and 391 and 183 Wt neurons on laminin were measured for neurite outgrowth. Analysis of variance shows Ko DRG cells growing longer neurites on astrocytes and laminin and this was highly significant for growth on astrocytes ( $p=0.0001$ ) but not on laminin ( $p=0.258$ ). Analysis of neurite outgrowth on laminin represents low n value and is reflected in the high p values.

On laminin, a difference between Ko and Wt neurite outgrowth was not seen at shorter time point (17-19 hours). The difference in neurite outgrowth between Ko and Wt neurons at 20-22 hours was significantly different ( $p = 0.05$ ). There was a tendency for neurites on laminin to break up during immunological labelling, a factor that particularly affected longer neurites. Hence fewer cells were measured on laminin (at T1 : 226 Ko and 97 Wt; and at T2 : 166 Ko and 86 Wt neurons) and these were not necessarily a representative sample especially at the longer time points. A gentler way of processing material on laminin, that preserved the full neurite morphology, would have to be found before the neurite outgrowth could be properly measured.

To further analyse the substrate effect I divided the data into two pools to enable me to maintain high n values. T1 in Fig 27 represents the pooled shorter time points (17-19 hours) and T2 in Fig 27 the pooled longer time points (20-22 hours). On astrocytes however, (Fig 27), the result is clear. Ko neurons grew longer neurites than Wt neurons at both time points. This was not significant at T1 ( $p = 0.08$ ), but at T2 was highly significant ( $p=0.0001$ ). These results are from 242 Ko and 278 Wt DRG neurons at T1 and 261 Ko and 295 Wt DRG neurons at T2 measured in a total of eight experiments. Thus at longer time points on astrocytes Wt (Thy-1 positive) neurite outgrowth was restricted but not inhibited.

When broken down into individual time points, the increasing tendency for Ko (Thy-1 negative) neurites to outgrow Wt (Thy-1 positive) ones on astrocytes becomes even clearer (Table 2). The significance values fluctuate due to the lower n values at 18, and especially 21 hours, but the difference by 22 hours is indisputable.

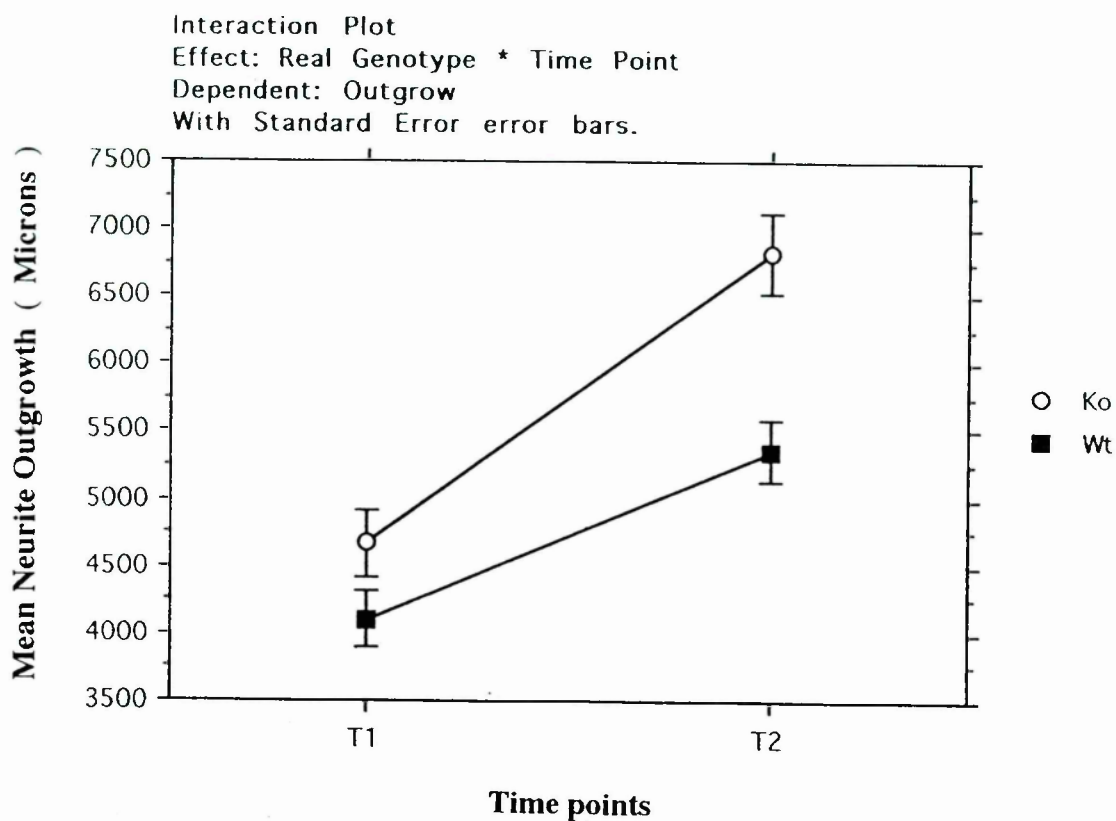
**Table 2**                      **Increased neurite outgrowth on astrocytes by Ko**  
**compared to Wt adult DRG neurons.**

<b>Time (Hours)</b>	<b>17</b>	<b>18</b>	<b>20</b>	<b>21</b>	<b>22</b>
<b>% difference <sup>a</sup></b>	12.9	13.8	18.2	27.2	34.7
<b>p value <sup>b</sup></b>	0.130	0.220	0.011	0.077	0.0001
<b>n Wt <sup>c</sup></b>	179	99	169	25	101
<b>n Ko <sup>c</sup></b>	142	100	126	35	100

<sup>a</sup> shows the percentage by which the mean neurite length from Ko (Thy-1 -ve) neurons exceeded that from Wt (Thy-1 +ve) DRG neurons.

<sup>b</sup> significance was determined by *t*-test using SuperAnova.

<sup>c</sup> Number of DRG neurons measured at each time point.



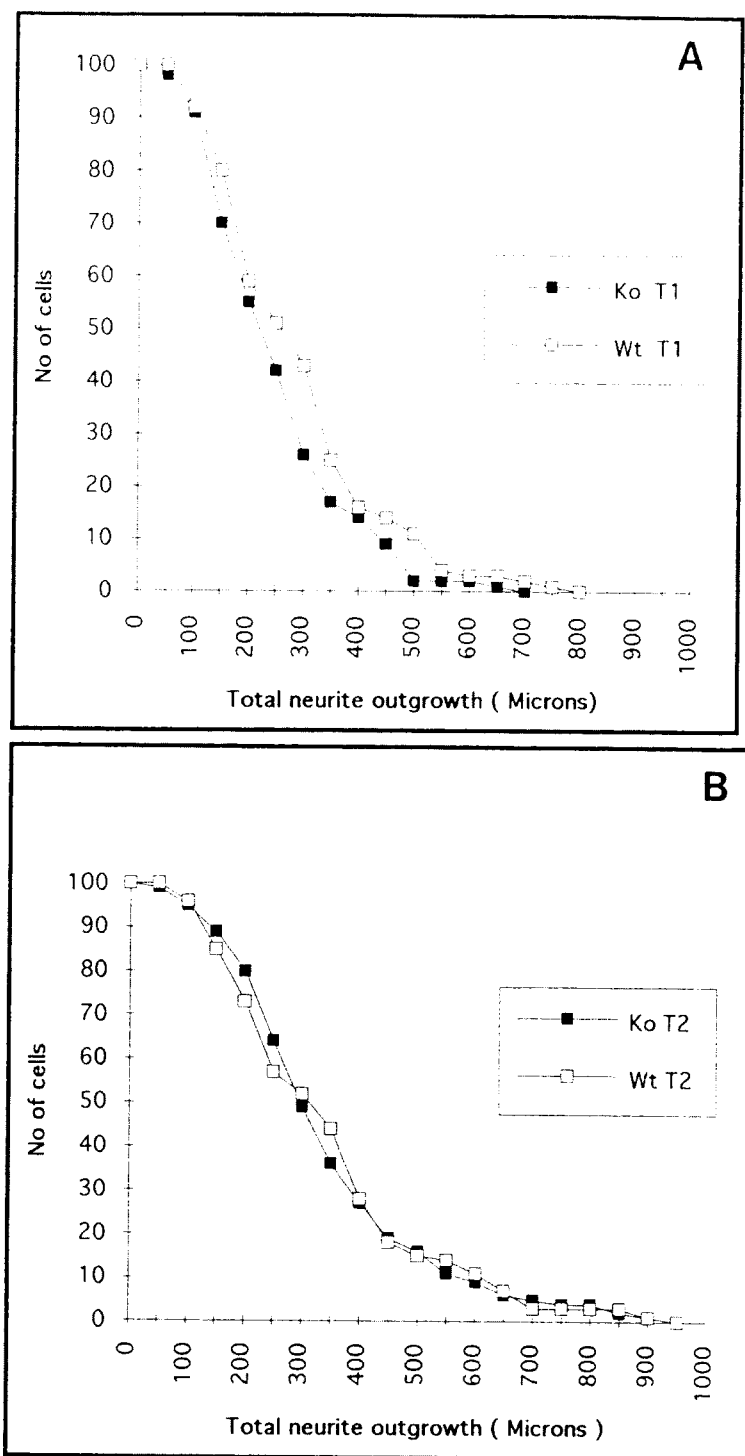
**FIG 27.** Neurite outgrowth of adult (P28) Thy-1 positive (Wt) and Thy-1 negative (Ko) DRG cells on astrocytes. Neurite outgrowth was measured at T1 (17-19 hours) and T2 (20-22 hours) in culture. Ko DRG cell neurites are longer than Wt DRG cell neurites at T1 and T2. The Ko neurons grow significantly longer neurites at T2 ( $p=0.0001$ ).

### **3.3.3 NEURITE OUTGROWTH FROM EMBRYONIC (E12) DRG NEURONS ON ASTROCYTES**

Embryonic DRG neurons are Thy-1 negative. As an internal control, I chose to test the neurite outgrowth of E12 DRG neurons from normal 129/Sv strain, and Thy-1 knockout mice. E12 DRG neurons were plated on laminin-coated dishes and on a monolayer of astrocytes. Cells were fixed after 18 hours for the Wt and 17 hours for the Ko neurons (this is referred to as T1). Both Ko and Wt neurons were fixed after 22 hours in culture (this is time point T2). The results (Fig 28 A and B) are from a single experiment. At the early time point, T1 (Fig 28 A) it appears that normal (Wt) 129/Sv neurons are growing marginally better than the Ko neurons; however, due to a misunderstanding, the Wt DRG neurons were fixed one hour later accounting for the slightly longer neurite outgrowth by the Wt DRG neurons. At the longer time point T2 (Fig 28 B), there is no difference in the neurite outgrowth of Ko and Wt cells. It is clear that embryonic (E12) Ko and Wt neurons grew very long bipolar or unipolar neurites but their total neurite outgrowth at 22 hours was less than adult cells because outgrowth from the adult DRG neurons was more complex. Clearly further experiments need to be done, but it appears from this single experiment that there is no significant difference between Ko and Wt embryonic neurons (when both are Thy-1 negative).

### **3.3.4 GROWTH OF DRG NEURONS ON A THREE-DIMENSIONAL COLLAGEN-ASTROCYTE SUBSTRATE**

Dorsal root ganglion axons grow readily on astrocyte monolayers (Fawcett *et al.*, 1992). Three-dimensional or organ cultures provide more accurate



**FIG 28.** Embryonic (E12) DRG cell neurite outgrowth on astrocytes. At time points T1 (T1=17-19 hours) (A) and T2 (T2=20-22 hours) (B) the neurite outgrowth in Ko and Wt DRG cells is similar. Wt and Ko DRG neurons extend neurites of similar length at all time points.

models of axonal behaviour *in vivo*. Fawcett (1989a) has shown that DRGs derived from postnatal animals are unable to penetrate three-dimensional cultures of mature astrocytes, while axons from embryonic DRGs and retina will grow for considerable distance through these cultures.

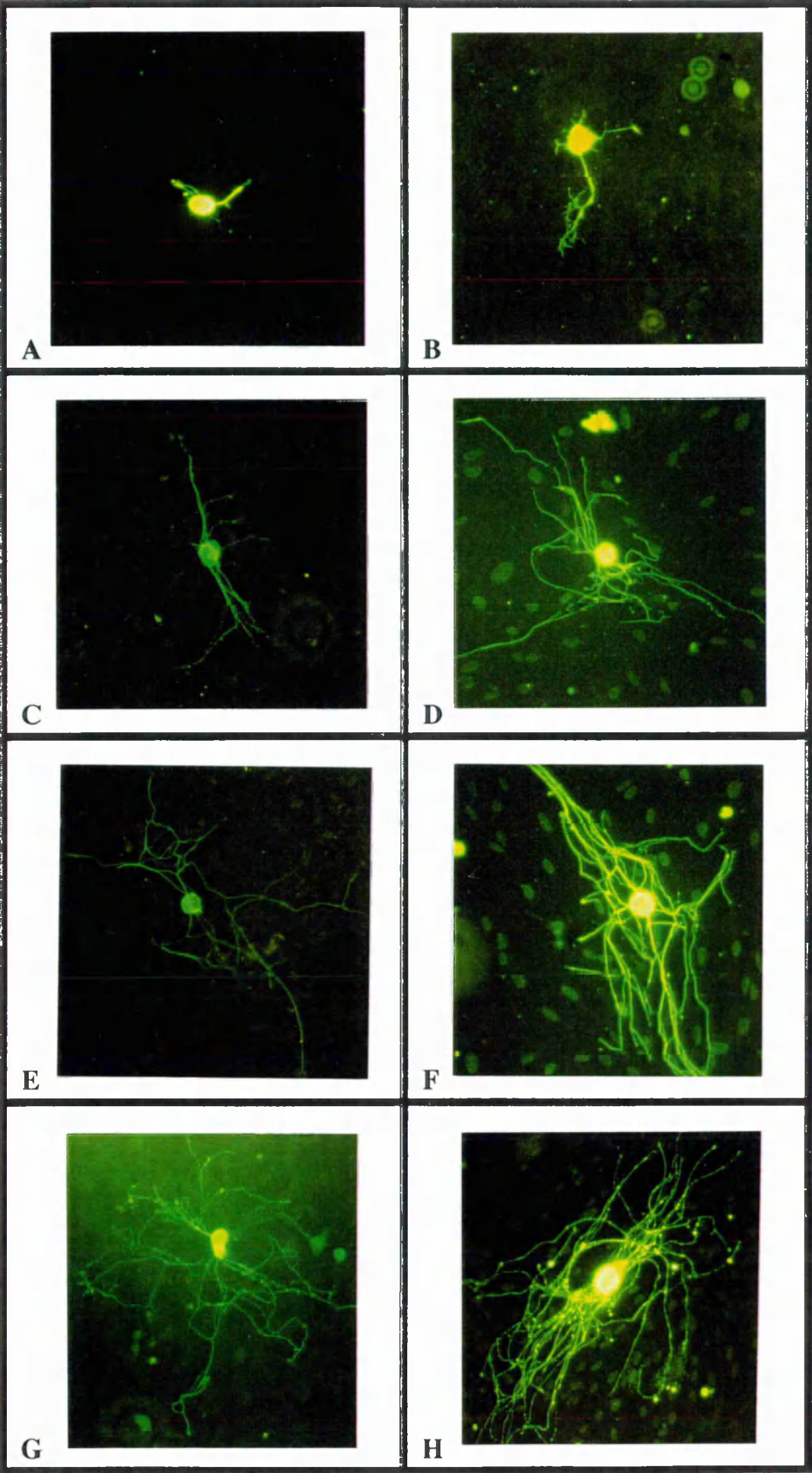
DRG neurons derived from Wt (+/+) and Ko (-/-) were grown in the astrocyte-DRG-astrocyte three-dimensional culture system.

DRG neurite growth was assessed at several time points (Fig 29 : 5 hr A and B; 18 hr C and D; 22 hr E and F and 48 hr G and H). On three-dimensional astrocyte sandwich, the DRG neurons extended shorter neurites at all time points compared to the growth on astrocyte monolayer (Fig 29 astrocyte sandwich A,C,E,G, astrocyte monolayer B,D,F,H ). In particular, it became possible to measure neurite outgrowth at 48 hours in the astrocyte sandwich system, whereas the longest time point possible with the astrocyte monolayer was 22 hours.

I compared the growth on three-dimensional astrocyte sandwich system of the F2 generation littermates. Qualitatively, in the three-dimensional culture system, at 18 hour (Fig 30 A and B) and at 22 hour (Fig 30 C and D) there was a strong impression that neurite outgrowth by Thy-1<sup>+/+</sup> (Fig 30 A,C) neurons was more restricted than that by Thy-1<sup>-/-</sup> neurons (Fig 30 B,D). The degree of fasciculation was greater and more frequent in the Thy-1<sup>+/+</sup> neurons compared to the Thy-1<sup>-/-</sup> neurons. I was unable to quantitate neurite outgrowth observations due to unavailability of the imaging system at this point in the project. The difference in growth between Thy-1<sup>-/-</sup> and Thy-1<sup>+/+</sup> became more apparent at longer time points, possibly due to two factors : firstly, differences between when individual neurons started to their neurites (typically 4-12 hours after introducing



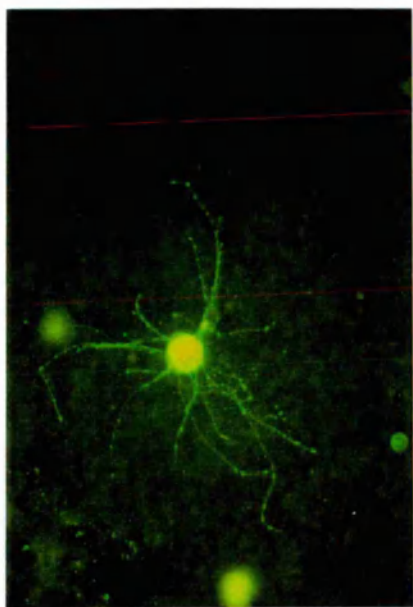
**FIG 29.** Growth of adult (Wt) DRG neurons in a three-dimensional astrocyte sandwich and astrocyte monolayer. Cells were fixed and then labelled with RT97 and TuJ 1 antibody at 5 hr (A,B), 18 hr (C,D), 22 hr (E,F) and 48 hr (G,H). FITC conjugated secondary antibody was used to visualise cells. At 48 hours, growth on the three-dimensional astrocyte sandwich (A,C,E and G) is restricted astrocyte monolayer (B,D,F and H) is complex. Bar 100 $\mu$ M.



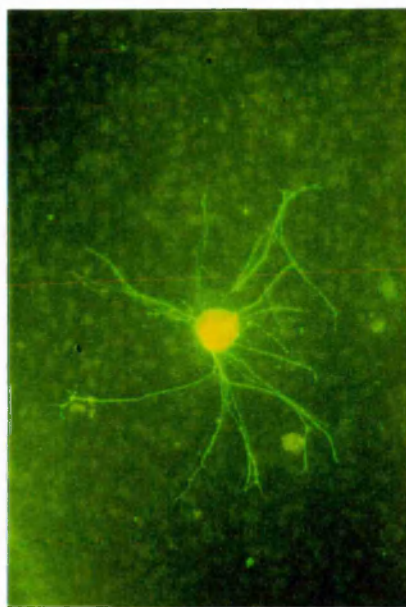
**FIG 30.** Growth at 18 hours (A,B) and 22 hours (C,D) of adult Wt (+/+) (A,C) and Knockout (-/-) (B,D) DRG neurons on three-dimensional astrocyte sandwich. Neurites are labelled with a cocktail of RT97 and TuJ 1 antibodies and visualised using FITC secondary antibody. Neurite outgrowth of wildtype neurons is restricted. Bar 100  $\mu$ M.

WILD TYPE

KNOCK OUT

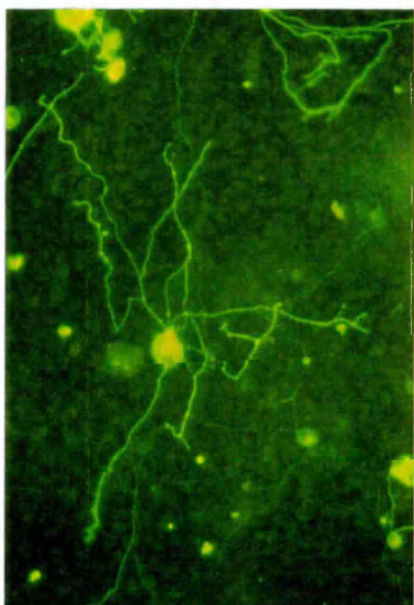


A

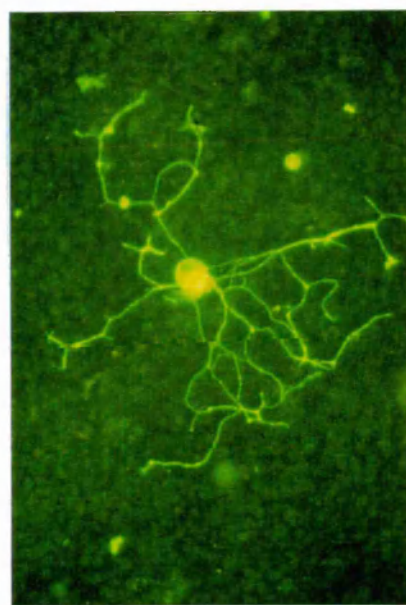


B

18hr



C



D

22 hr

to culture) became less significant at longer time points; secondly, the Thy-1 signalling system in the neurites might be more effective after the cells have recovered from the trauma of being introduced into culture.

### **3.3.5                    NEURITE OUTGROWTH OF ADULT DRG** **NEURONS ON MAG EXPRESSING COS CELLS**

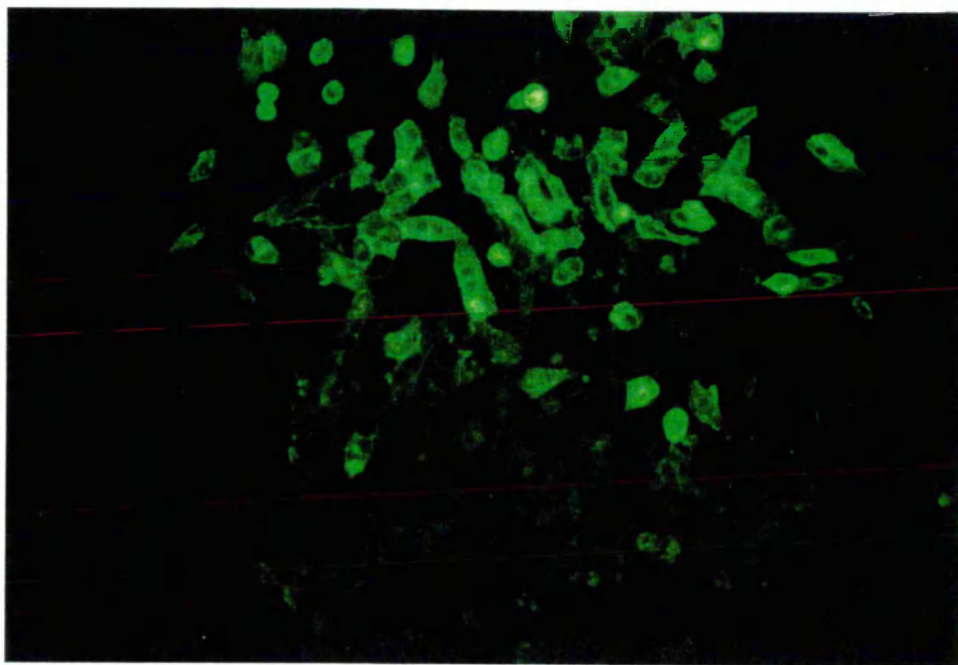
Neurite growth of neonatal and older cerebellar neurons, (Mukhopadhyay *et al.*, 1994) postnatal retinal, hippocampal, superior cervical ganglion, spinal and adult DRG (DeBellard *et al.*, 1996) neurons is inhibited when grown on a substrate that expresses MAG. Since this would give a useful point of comparison with the partial inhibition of neurite seen with Thy-1, we assessed how Thy-1<sup>+/+</sup> and Thy-1<sup>-/-</sup> neurons derived from adult mouse would grow over monolayers of COS cells either expressing, or not expressing MAG.

The first thing we noticed is shown in Fig 31. Labelling of the COS cell monolayers with an anti-MAG antibody showed that not all the cells expressed detectable levels of MAG. Islands of non-expressing cells dotted the monolayer (Fig 31 A) and it was on these monolayers alone that adult DRG cells adhered and grew neurites until they encountered MAG-expressing COS cells. On no occasion did we see neurites from adult DRG neurons grow over MAG expressing COS cells. When the total neurite length of DRG cells adherent to the MAG expressing islands on COS cell monolayer were measured, they were about half as long as the growth of neurites over the islands of "control", non-expressing COS cells. The more notable factor was that the number of adult DRG neurons adherent to the MAG-expressing COS monolayer was about 10% the level adherent to the "control" COS cells, which in turn was comparable to the numbers of cells adherent to astrocytes.

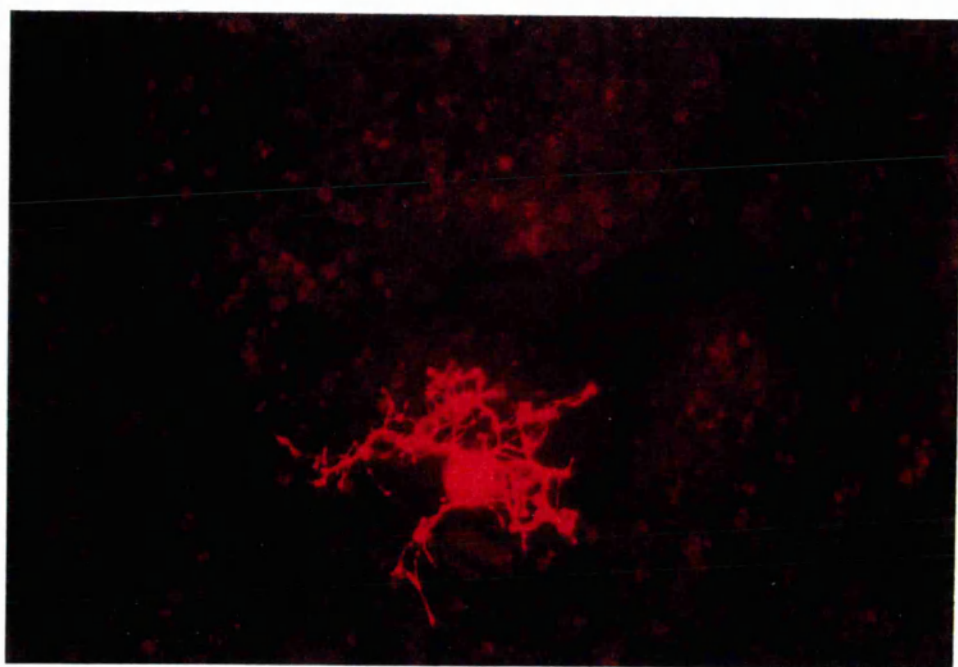
On RC2, the control substrate, 60% more Thy-1<sup>-/-</sup> neurons were extending neurites when compared with MAG expressing substrate. At 18 hours on the MAG-expressing COS cells the neurite length was no longer then 2500  $\mu$ M but on control RC2 cells at the same time point, neurites measuring 6000  $\mu$ M were not unusual. Both Thy-1<sup>+/+</sup> and Thy-1<sup>-/-</sup> adult DRG cells were plated onto MAG-expressing COS cells. When the neurite outgrowth of more than 100 cells of each type was measured after 18 and 22 hours, no difference was seen between neurite outgrowth from Thy-1<sup>+/+</sup> and Thy-1<sup>-/-</sup> adult DRG neurons. From these results I deduced that Thy-1 plays no part in the inhibition of neurite outgrowth of adult DRG cells in response to MAG. Unfortunately, these results disappeared when the computer / image analysis system was stolen and only a partial quantitative record survives.

**FIG 31.** Adult (Wt) DRG neurons on MAG expressing substrate. When labelled with anti-MAG antibody (FITC secondary antibody), I observed patches of non-MAG expressing cells (A) and the adult DRG neurons (GAP43labelled, TRITC secondary antibody) grew preferentially on areas on substrate which showed no MAG expression (B). Bar 100  $\mu$ M.





A



B



## **CHAPTER 4**

### **DISCUSSION**

In normal adult CNS, synaptic connections in the grey matter are relatively stable, but a degree of plasticity allowing synaptic rearrangement is retained. This suggests that the axonal growth in terminal fields is regulated and not totally prohibited allowing a limited degree of re-growth. Cell adhesion molecules that increase cell motility, neurite outgrowth and defasciculation during development might enable new synapses to form or to facilitate synaptic remodelling in postnatal life. Cell adhesion molecules that promote cell aggregation and fasciculation, decrease motility and neurite outgrowth might promote consolidation of appropriate synaptic structures in association with activity dependent modifications (Doherty *et al.*, 1995).

Complex axonal-astrocyte interactions form an integral part of regulation of axonal growth in the terminal fields. Lack of ability of an axon to grow in an environment is a product of glial and neuronal effects (Fawcett, 1992). The state of maturation of the glia and axons determines the ability to re-grow. Young axons can grow on mature glia and adult axons will grow on young glia (Muller and Best, 1989; Rosario *et al.*, 1993) but regeneration of adult (Thy-1 positive) axons in a mature astrocytic environment is not possible partly due to an over-riding effect of inhibitory molecules over growth promoting molecules.

Neurite outgrowth studies of neurons derived from embryonic and early post-natal material have been useful in contributing to our knowledge of the multifactorial

system that regulates growth (Ard and Bunge, 1988; Smith-Thomas *et al.*, 1994; Felsenfeld *et al.*, 1994., Luo *et al.*, 1993., Faivre-Sarrailh and Rougon, 1997) and the complex receptor system that operates in the glial environment (Doherty and Walsh, 1994). To identify the relevance of these results to regeneration in adult is impossible. More meaningful conclusions can only be derived by examining the mature astrocyte-adult axon paradigm. This I have addressed in the present study.

It has previously been demonstrated in this laboratory that the neuronal glycoprotein Thy-1 can function as a growth restricting molecule on astrocytes (Tiveron *et al.*, 1992 and 1994). That work was done on a transfected neural cell line. Given the availability of knockout mice I set out to assess whether Thy-1, a major glycoprotein of the surface of mature but not growing axons (Xue *et al.*, 1991), is able to restrict neurite outgrowth from real axons grown from primary cultured DRG neurons over a monolayer of astrocytes. I compared extent of axonal growth from normal, mature Thy-1 expressing DRG neurons with DRG neurons from mice in which the Thy-1 gene has been deleted by homologous recombination. I used a monolayer of mature astrocytes to study how mature neuron-astrocyte interaction restricts neurite outgrowth and limits axonal regeneration. An image analysis system was used to measure neurite outgrowth.

Using this system, I have confirmed the growth restricting effect of Thy-1 on astrocytes. Whilst the neurite outgrowth of Wt (Thy-1 positive) adult DRG cells was not inhibited dramatically, I did see a significant restriction of neurite outgrowth when compared to Ko Thy-1 negative adult DRG cells. Analysis of growth from embryonic neurons (taken before they express Thy-1 in normal mice) showed the 129/Sv and Thy-1 Ko neurons did not differ significantly. This important control shows that Ko and Wt neurons do not inherently differ in their neurite outgrowth over astrocytes before Thy-1 is expressed. These results support the hypothesis that Thy-1 might down regulate (but not totally prohibit) axonal growth on astrocytes. Thy-1 may be one (but not the only)

member of a multifactorial system resulting in relatively mild regulation of axonal growth on mature astrocytes. I saw Ko (Thy-1 negative) DRG cells growing better than Wt (Thy-1 positive) on laminin as well, which may indicate that Thy-1 may be interacting with an integrin type receptor on the axon, although this result should at this stage be treated with caution.

I have improved the isolation procedure for deriving maximally viable adult DRG neurons which when introduced into culture result in reproducible neurite growth that is approximately 6-fold longer than what I obtained using other published procedures (Lindsay, 1989). Using DRG neurons to study neurite outgrowth has proved to be a complex and unwieldy assay system. From adult neurons the growth is immensely bifurcating, a pattern that precludes meaningful analysis by time-lapse examination of individual growth cones on cells that can acquire up to 50 growth cones. Initiation of neurite outgrowth by adult neurons is staggered over the period 4-24 hours and some even longer after plating out, in comparison with embryonic neurons most of which commence growth by 4 hours. This staggered initiation of growth of adult DRG neurons masks differences in growth rate unless hundreds of cells are measured, and the complexity of growth means that total neurite length cannot be measured at times greater than 22 hours after plating. Whilst my results do demonstrate a growth restrictive effect of Thy-1 on a monolayer of astrocytes, I have devised a convenient three-dimensional culture system, which may more closely recapitulate the *in vivo* astrocyte environment in which outgrowing neurites are sandwiched between adhering layers of astrocytes. Neurite outgrowth under these conditions is slower, a wider spectrum of DRG cells of all sizes have the opportunity to grow axons over a longer time span and the neurites have a simpler pattern of growth than on a monolayer of astrocytes thus lending itself as a more practical system for analysis.

Thy-1 on cell surface is highly resistant to proteolysis, presumably due in part to its GPI anchorage. This also means that Thy-1 does not span the surface membrane and in order to down-regulate growth it presumably interacts with a transmembrane protein. The latter may be sensitive to trypsinisation. If this is the case, Thy-1 inhibition will not be effective until the transmembrane protein has regenerated on the surface. The lack of any effect of Thy-1 at shorter time points between Ko and Wt DRG cells may reflect this refractory period. Since Thy-1 function is dependent on its interacting with a transmembrane signalling molecule, it is only after the cell surface is restored and remodelled do we see a difference in neurite outgrowth. After 17 hours, when it was assumed most but not all of the cells which were capable of growing neurites had started growing neurites we were able to see a measurable restriction of neurite outgrowth on astrocytes in Wt DRG cells. However, although most meaningful results may be derived after 24 hours of neuritic growth (a time when most of the neurons capable of growing neurites are extending neurites) the complexity of neurite network made measurement of neurite outgrowth using the present system difficult. The three-dimensional system facilitates the study of larger percentage of the population of DRG neurons contributing to neurite outgrowth.

Further analysis of neurite outgrowth in DRGs derived from control mice in which appropriate neuronal expression of Thy-1 has been restored transgenically would have tested more stringently whether the effects observed are due the absence of Thy-1, and not due to an alteration in a closely linked gene. I would also like to have had a better range of controls available to distinguish an effect due to Thy-1 from other possible factors. As an optimum control, I would like to have studied neurite outgrowth from animals in which there has been restoration of Thy-1 expression using transgenic methods to the identical mice in which the endogenous Thy-1 gene has been inactivated.

Given the multifactorial nature of regulation of axonal growth on astrocytes, it may be difficult to demonstrate the effect of any one component *in vivo*, whereas in culture a difference in rate of growth due the absence of a targeted molecule can be measured. We know that Thy-1 is one of many candidate molecules that regulates axonal growth of adult neurons. Studies of growth promoting effects of N-cadherin, L-1, NCAM and integrins have also reported a mild effect on neurite outgrowth on astrocytes due to any one receptor system (Neugebauer *et al.*, 1988; Tomaselli *et al.*, 1988) and argued for a multifactorial regulation of growth on astrocytes. If several interactions regulate axonal growth, then eliminating one of them, as in a knockout paradigm might, fail to show failure of expected regulation due to the action of remaining systems (Cremer *et al.*, 1994). This is suggested by the absence of effect on axonal growth in tenascin (Saga *et al.*, 1992) and NCAM (Doherty and Walsh, 1992) knockout mice *in vivo*, despite very extensive literature showing the importance of these molecules in regulating axonal growth *in vitro*. *In vivo* experiments show that axonal regeneration in the CNS of adult myelin-associated glycoprotein deficient mice was not improved when compared to wild-type animals. *In vivo* results do not support the functional role of myelin associated glycoprotein as a potent inhibitor of axonal regeneration in the adult mammalian CNS (Bartsch, 1996). However, depending on age and type of neuron, on two-dimensional cultures, MAG is very effective at restricting axonal growth (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994). Similarly, it is probable that Thy-1 effect is relatively minor *in vivo*, if it is just detectable *in vitro*. Overall my result suggest that Thy-1 may play a role in a complex system of cell surface molecule-ligand interactions to dictate the growth pattern of adult axons.

## REFERENCES

- Anderton, B.H., Breinburg, D., Downes, M.J., Green, P.J., Tomlinson, J., Ulrich, J., Wood, J.N. and Kahn, J. (1982). Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. *Nature* **298**: 84-86.
- Appel, F., Holm, J., Conscience, J., Halbach, F., Fassiner, A., James, P. and Schachner, M. (1995). Identification of the border between Fibronectin Type III homologous repeats 2 and 3 of the neural cell adhesion molecule L1 as a neurite outgrowth promoting and signal transduction domain. *J Neurobiol* **28**: 297-312.
- Ard, M.D. and Bunge, R. (1988). Heparan Sulphate Proteoglycans and Laminin immunoreactivity on cultured astrocytes: Relationship to differentiation and neurite growth. *J Neurosci* **8**: 2844-2858
- Attardi, D.G. and Sperry, R.W. (1963). Preferential selection of central pathways by regenerating optic fibers. *Exp Neurol* **7**: 46-64.
- Bandtlow, E., Schmidt, M.F., Hassinger, T.D., Schwab, M.E. and Kater, S.B. (1993). Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. *Science* **259**: 80-83.
- Barbacid, M., Lamballe, F., Pulido, D. and Klein, R. (1991). The Trk family of tyrosine protein kinase receptors. *Biochimica et Biophysica Acta* **1072**: 115-127.
- Barde, Y. A. (1989). Trophic factors and neuronal survival. *Neuron* **2**: 1525-1534.
- Barde, Y -A., Edgar, D. and Thoenen, H. (1980). Sensory neurons in culture: changing requirements for survival factors during embryonic development. *Proc. Natl. Acad. Sci* **77**: 1199-1203.
- Bartsch, U. (1996). Myelination and axonal regeneration in the central nervous system of mice deficient in the myelin-associated glycoprotein. *J Neurocytol.* **25**: 303-313.

- Bastmeyer, M., Bahr, M. and Stuermer, C. A. (1993). Fish optic nerve oligodendrocytes support axonal regeneration of fish and mammalian retinal ganglion cells. *Glia* **8**: 1-11.
- Bedi, K.S., Winter, J., Berry, M. and Cohen, J. (1992). Adult rat dorsal root ganglion neurons extend neurites on predegenerated but not on normal peripheral nerves *in vitro*. *Eur. J. Neurosci* **4**: 193-200.
- Benfey, M. and Aguayo, A.J. (1982). Extensive elongation of axons from rat brain into peripheral nerve grafts. *Nature* **296**: 150-152.
- Bergman, B.S., Kunkel-Bagden, E., Schnell, L., Ling-Dai, H., Gao, D. and Schwab, M.E. (1995). Recovery from spinal cord injury mediated by neurite growth inhibitors. *Nature* **378**: 498-501.
- Bixby, J.L. and Harris, W.A. (1991). Molecular mechanisms of axon growth and guidance. *Ann Rev Cell Biol* **7**: 117-159.
- Björklund, A. and Stenevi, U. (1984). Intracerebral neural explants: neural replacement and reconstruction of damaged circuitries. *Ann Rev Neurosci* **7**: 279-308.
- Björklund, A., Lindvall, O., Isacson, O., Brundin, P., Wictorin, K *et al* ., (1987). Mechanism of action of intracerebral neural implants : Studies on nigral and striatal grafts to the lesioned striatum. *Trends Neurosci* **10**: 509-516.
- Blankenhorn, E.P. and Douglas, T.C. (1972). Location of the gene for theta antigen in the mouse. *J Hered.* **63**: 259-263.
- Bozyczko, D. and Horwitz, A.F. (1986). The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. *J Neurosci* **6**: 1241-1251.
- Bray, G. M., Rasminsky, M. and Aguayo, A.J. (1981). Interactions between axons and their sheath cells. *Annu Rev Neurosci* **4**: 127-162.

- Brittis, P. A., Canning, D.R. and Silver, J. (1992). Chondroitin sulfate as a regulator of neuronal patterning in the retina. *Science* **255**: 733-736.
- Brook, G. A., Lawrence, J. M., Shah, B. and Raisman, G. (1994). Extrusion transplantation of Schwann cells into the adult rat thalamus induces directional host axon growth. *Exp Neurol* **126**: 31-43.
- Cajal, R.S. (1928). *Degeneration and Regeneration of the Nervous System*. Vol II. Hefner Publishing Co. New York.
- Carlstedt, T., Culheim, S., Risling, M. and Ulfhake, B. (1989). Nerve fibre regeneration across the PNS-CNS interface at the root spinal cord junction. *Brain Res Bull* **22**: 93-102.
- Caroni, P. and Schwab, M.E. (1988) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J Cell Biol* **106**: 1281-1288.
- Coggeshall, R. E. and Youngblood, C.S. (1983). Recovery from spinal transection in fish; regrowth of axons past the transection. *Neurosci Lett*. **38**: 227-231.
- Cohen, F.E., Novotny, J., Sternberg, M.J.E., Campbell, D.G. and Williams A.F. (1981). Analysis of structural similarities between brain Thy-1 antigen and immunoglobulin domains: Evidence for an evolutionary relationship and a hypothesis for its functional significance. *Biochem J*. **195**: 31-40.
- Cohen, J., Burne, F.J., Winter, J. and Bartlett, P. (1986). Retinal ganglion cells lose their response to laminin with maturation. *Nature* **322**: 465-467.
- Cohen, J. and Johnson, A.R. (1991). Differential effects of laminin and merosin on neurite outgrowth by developing retinal ganglion cells. *J Cell Sci Suppl* **15**: 1-7.
- Colamarino, S. A. and Tessier -Lavigne, M (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell* **81**: 621-629.



Collins, F. and Lee, M.R. (1982). A reversible developmental change in the ability of ciliary ganglion neurons to extend neurites in culture. *J Neuroscience* **2**: 424-430.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D.M. and Stanfield, B.B. (1985).

Regressive events in neurogenesis. *Science* **225**: 1258-1265.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kreamer, P., Scheff, S., *et al* . (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* **367**: 455-459.

Cunningham, B.A., Hemperley, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R. and Edelman, G.M. (1987). Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* **236**: 799-806.

David, S. and Aguayo, A. J. (1981). Axonal elongation into peripheral nervous system bridges after CNS injury in adult rats. *Science* **241**: 931-933.

Davies, S.J.A., Field, P.M. and Raisman, G. (1993). Long fibre growth by axons of embryonic mouse hippocampal neurons microtransplanted into the adult rat fimbria. *Eur J Neurosci* **5**: 95-106.

Davies, A.M. (1988a). Role of neurotrophic factors in normal development. *Trends Genet* **4**: 139-143.

DeBellard, M-E., Tang, S., Mukhopadhyay, G., Shen, Y-J. and Filbin, M.T. (1996). Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein. *Mol Cell Neurosci*. **7**: 89-101.

Doherty, P. and Walsh, F.S. (1992). Cell adhesion molecules, second messengers and axonal growth. *Curr Opin Neurobiol*. **2**: 595-601.

Doherty, P. and Walsh, F.S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. *Curr Opin Neurobiol* **4**: 49-55.

- Doherty, P., Fazeli, M.S. and Walsh, F.S. (1995). The neural cell adhesion molecule and synaptic plasticity. *J Neurobiology* **26**: 437-446.
- Dunnett, S. B., Low, W. C., Iverson, S. D., Stenevi, U. and Björklund, A. (1982). Septal transplants restore maze learning in rats with fimbria-fornix lesions. *Brain Res* **251**: 335-348.
- Faivre-Sarrailh, C and Rougon, G. (1997). Axonal molecules of the immunoglobulin superfamily bearing a GPI anchor: their role in controlling neurite outgrowth. *Mol Cell Neurosci* **9**: 109-115.
- Fawcett, J. W., Housden, E., Smith, T.L. and Meyer, R.L. (1989a). The growth of axons in three-dimensional astrocyte cultures. *Dev Biol* **135**: 449-458.
- Fawcett, J.W., Rokos, J. and Bakst, I. (1989b). Oligodendrocytes repel axons and cause axonal growth cone collapse. *J Cell Sci* **92**: 93-100.
- Fawcett, J.W. (1992). Intrinsic neuronal determinants of regeneration. *TINS* **15**: 5-8.
- Fawcett, J.W., Fersht, N., Housden, L., Schavhner, M. and Pesheva, P. (1992). Axonal growth on astrocytes is not inhibited by oligodendrocytes. *J.Cell.Sci.* **102**: 571-579.
- Felsenfeld, D.P., Hynes, M.A., Skoler, K.M., Furley, A. J. and Jessell, T.M. (1994). Tag-1 can mediate homophilic binding, but neurite outgrowth on TAG-1 requires an L1-like molecule and  $\beta 1$  integrins. *Neuron* **12**: 675-690.
- Galileo, D.S., Majors, J., Horwitz, A. F. and Sanes, J.R. (1992). Retrovirally induced antisense integrin RNA inhibits neuroblast migration. *Neuron* **9**: 1117-1131.
- Golding, J. P., Shewan, D., Berry, M. and Cohen, J. (1996). An *in vitro* model of the rat dorsal root entry zone reveals developmental changes in the extent of sensory neuron growth into the spinal cord. *Mol Cell Neurosci* **7**: 191-203.
- Grafstein, B. (1986). Regeneration in ganglion cells. *In* The Retina. R, Adler., and D, Farber, Eds: 275-335. Academic Press. USA.

- Hatten, M.E., Fishell, F., Stütt, T.N. and Mason, C.A. (1990). Astroglia as a scaffold for development of the CNS. *Seminars. Neurosci* **2**: 455-456.
- Heumann, R., Korsching, S., Bandtlow, C. and Thoenen, H. (1987). Changes of nerve growth factor synthesis in non-neuronal cells in response to sciatic nerve transection. *J Cell Biol* **104**: 1623-1631.
- Hökfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G. and Arimura, A. (1976). Immunocytochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. *Neurosciences* **1**: 131-136.
- Holder, N. and Clarke, J. D. (1988). Is there correlation between continuous neurogenesis and directed axon regeneration in the vertebrate nervous system? *Trends Neurosci* **11**: 94-99.
- Igarashi, M., Strittmatter, S.M., Vertanian, T. and Fishman, M.C. (1993). Mediation by G-proteins of signals that cause collapse of growth cones. *Science* **259**: 77-79.
- Iwashita, Y., Kawaguchi, S. and Murata, M. (1994). Restoration of function by replacement of spinal cord segments in the rat. *Nature* **367**: 167-170
- Jakeman, L.B. and Reier, P.J. (1991). Axonal projections between foetal spinal cord transplants and the adult rat spinal cord: a neuroanatomical tracing study of local interactions. *J Comp Neurol* **307**: 311-334.
- Jessell, T.M. and Dodd, J. (1986). Neurotransmitters and differentiation antigens in subsets of sensory neurons projecting to the spinal dorsal horn. *In* *Neuropeptides in Neurologic and Psychiatric Diseases*. Martin, J.B., and Barchas, J.D. Ed. p. 111-133. Raven Press. N.Y.
- Johnson, A.R., Wigley, C.B., Gregson, N.A., Cohen, J. and Berry, M. (1988). Neither laminin nor prior optic nerve section are essential for the regeneration of adult mammalian retinal ganglion cells *in vitro*. *J Neurocytol* **17**: 95-104.

Johnson, A.R. (1993). Contact inhibition in failure of mammalian CNS axonal regeneration. *Bioessays* **15**: 807-813.

Johnson, E.M., Gorin, P.D., Brandeis, L.D. and Pearson, J. (1980). Dorsal root ganglion neurons are destroyed by exposure *in utero* to maternal antibody to nerve growth factor. *Science* **210**: 916-918.

Johnson, E.M., Rich, K.M. and Yip, H.K. (1988). The role of NGF in sensory neurons *in vivo*. *TINS* **9**: 33-37.

Kennedy, T. E., Serafini, T., De la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrines are diffusible chemotrophic factors for commissural axon in the embryonic spinal cord. *Cell* **12**: 425-235.

Kierstead, S.A., Rasminsky, M., Fukuda, Y., Carter, D.A., Aguayo, A.J. and Vidal-Sanz, M. (1989). Electrophysiologic responses to hamster superior colliculus evoked by regenerating retinal axons. *Science* **246**: 255-257.

Kromer, L.F. and Carson, J. (1985). Transplants of Schwann cell cultures promote axonal regeneration in the adult mammalian brain. *Proc Natl Acad Sci USA*. **82**: 6330-6334.

Lander, A.D., Fujii, D.K. and Reichardt, L.F. (1985). Laminin is associated with the neurite outgrowth promoting factors in conditioned media. *Proc Natl Acad Sci USA*. **82**: 2183-2187.

Lawson, S.N., Harper, E.I., Harper, A.A., Garson, J.A. and Anderton, B.H. (1984). A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurons. *J Comp Neurol* **228**: 263-272

Lawson, S.N. and Waddell, P.J. (1991). Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. *J Physiol* **435**: 41-63.

Le Dourain, N. and Smith, J. (1988). Development of the peripheral nervous system from the neural crest. *Ann Rev Cell Biol* **4**: 375-404.

- Ledbetter, J.A. and Herzenberg, L.A. (1979). Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunological Rev* **47**: 63-90.
- Lefcort, F., Clary, D.O., Rusoff, A.C. and Reichardt, L.F. (1996). Inhibition of the NT3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. *J Neurosci* **16**: 3704-3713.
- Leibrock, J., Lottspeich, F., Hohn, A., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.A. (1989). Molecular cloning and expression of BDNF. *Nature* **341**: 149-155.
- Li, Y. and Raisman, G. (1995). Sprouts from cut corticospinal axons persist in the presence of astrocytic scarring in long-term lesions of adult rat spinal cord. *Exptl Neurol* **134**: 102-111.
- Lindsay, R.M., Barde, Y-A., Davies, A.M. and Rohrer, H. (1985a). Differences and similarities in the neurotrophic requirement of sensory neurons derived from the neural crest and neural placode. *J Cell Sci Suppl* **3**: 115-129.
- Lindsay, R. M. (1988). Brain-derived neurotrophic factor: an NGF related neurotrophin. *In* *Neurotrophic Factors*. Ed., S. E. Loughlin and J. H. Fallon. Academic Press. N Y.
- Lindsay, R.M. (1988a). Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J Neurosci* **8**: 2394-2404.
- Lindsay, R.M. and Harmar, A.J. (1989). Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* **337**: 362-364.
- Lindsay, R.M., Lockett, C., Sternberg, J. and Winter, J. (1989). Neuropeptide expression in cultures of adult sensory neurons: modulation of Substance P and Calcitonin Gene-Related peptide levels by nerve growth factor. *Neurosci* **33**: 53-65.
- Lipton, S.J., Levin, L.A. and Barnstable, C.J. (1988). Various Thy-1 antibodies differentially enhance outgrowth by rodent retinal ganglion cells. *Soc Neurosci Abstr* **14**: p747.

Lisanti, M.P., Scherer, P.E., Tang, Z. and Sargiacomo, M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *TICB* **4**: 231-235.

Liuzzi, F.J. and Lasek, R.J. (1987). Astrocytes block axonal regeneration in mammals by activating the physiological pathway. *Science* **237**: 642-645.

Lochter, A., Vaughan, L., Kaplony, A., Prochiantz, A., Schachner, M. and Fassiner, A. (1991). J1/Tenascein in substrate-bound and soluble form displays contrary effects on neurite outgrowth. *J Cell Biol* **113**: 1159-1171.

Luo, Y., Raible, D. and Raper, J.A. (1993). Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**: 217-227.

Mahanthappa, N.K. and Patterson, P.H. (1987). Antibodies to Thy-1 promote neurite outgrowth from rat sympathetic neurons *in vitro*, and promote neurite initiation by rat adrenal chromaffin and PC12 cells in the absence of NGF. *Soc Neurosci Abstr* **13**: 5.

Mason, E. and Williams, A. (1980). The Kinetics and binding to membrane antigens in solution and at the cell surface. *Biochem Journ* **187**: 1-20.

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J. and Braun, P.E. (1994). Identification of myelin associated glycoprotein as a major myelin-derived inhibitor of neurite outgrowth. *Neuron* **13**: 805-811.

McLoon, S.C. and Lund, R.D. (1983). Development of foetal retina, tectum and cortex transplanted to the superior colliculus of adult rats. *J Comp Neurol* **217**: 376-389.

McMahon, S.B. (1992). *In* Sensory Neurons, Diversity, Development and Plasticity. Ed Scott, S.A. Oxford University Press, New York and Oxford, pp 333-362.

Memberg, S.P. and Hall, A.K. (1996). Proliferation, differentiation, and survival of rat sensory neuron precursors *in vitro* require specific trophic factors. *Mol Cell Neurosci* **6**: 323-335.

Messersmith, E.K., Leonardo, E.D, Shatz, C.J., Tessier-Lavigne, M., Goodman, C.S. and Kolodkin, A.L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* **14**: 949-959.

Minichiello, L., Piehl, F., Vazquez, E., Schimmang, T., Hockfelt, T., Reres, J. and Klein, R. (1995). Differential effects of combined Trk receptor mutations on dorsal root ganglion and inner ear sensory neurons. *Development* **121**: 4067-4075.

Moody, S. A., Quigg, M. and Frankfurter, A. (1989). Development of the peripheral trigeminal system in the chick revealed by an isotype-specific anti-beta-tubulin monoclonal antibody. *J of Comp Neurol* **279**: 567-580.

Moos, M., Tacke, R., Scherer, H., Teplow, D., Fruh, K. and Schachner, M. (1988). Neural cell adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* **334**: 701-703.

Morris, R.J. (1985). Thy-1 in the developing nervous tissue. *Dev Neurosci* **7**: 133-160.

Morris, R.J., Beech, N.J., Barber, P.C. and Raisman, G. (1985a). Early stages of Purkinje cell maturation demonstrated by Thy-1 immunohistochemistry on postnatal rat cerebellum. *J.Comp Neurol* **14**: 427-452.

Morris, R. (1992). Thy-1, the enigmatic extrovert on the neuronal surface. *BioEssays* **14**: 715-722.

Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R. and Filbin, M.T. (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13**: 757-767.

Muller, C.M. and Best, J. (1989). Ocular dominance plasticity in adult cat visual cortex after transplantation of cultured astrocytes. *Nature* **342**, 427-430.

Needels, D.L., Nieto-Sampedro, M. and Cotman, C.W. (1986). Induction of neurite-promoting factor in rat brain following injury or deafferentation. *Neuroscience* **18**: 517-526.

- Neugebauer, K.M., Tomaselli, K.J., Lilien, J. and Reichardt, L.F. (1988). N-Cadherin N-CAM and integrins promote retinal neurite outgrowth on astrocytes *in vitro*. *J Cell Biol* **107**: 1177-1187.
- Pesheva, P., Spiess, E. and Schachner, M. (1989). J1-160 and J1-180 are oligodendrocyte secreted non-permissive substrates for cell adhesion. *J Cell Biol* **109**: 1765-1778.
- Pindzola, R.R., Doller, C. and Silver, J. (1993). Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Devl Biol* **156**: 34-48.
- Poltark, M., Sadoul, R., Keilhauer, G., Landa, C., Fahrig, T. and Schachner, M. (1987). Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. *J Cell Biol* **105**: 1893-1899.
- Pons, T.P., Garraghty, P.M., Ommaya, A.K., Kass, J.H., Taub, E. and Miskin, M. (1991). Massive cortical reorganisation after sensory deafferentation in adult macaques. *Science* **252**: 1857-1860.
- Porter, B.E. and Sanes, J.R. (1995). Distinct adhesive properties of ciliary and choroid neurons from the avian ciliary ganglion. *J. Neurobiol* **28**: 381-390.
- Price, J. An immunohistological and quantitative examination of dorsal root ganglion neuronal subpopulations. (1985). *J. Neurosci.* **5**: 2051-2059.
- Purves, D. (1988). *Body and brain: A trophic theory of neural connections*. Harvard Univ Press. Cambridge. p.124-141.
- Raisman, G. and Ebner, F.F. (1983). Mossy fibre projections into and out of hippocampal transplants. *Neuroscience* **9**: 783-801.
- Raisman, G. (1985). Specialised neuro-glial arrangement may explain the capacity of vomeronasal axons to reinnervate central neurons. *Neurosci* **14**: 237-254.



- Ranson, S. W. (1912). Degeneration and regeneration of nerve fibres. *J. Comp. Neurol.* **22**: 487-545.
- Reier, P.J., Stensaas, L.J. and Guth, L. (1983). The astrocytic scar as an impediment to regeneration in the central nervous system. In *Spinal cord Reconstruction*, Ed C. C. Kao, R.P. Bunge and P.J. Reier, pp 163-195. New York. Raven Press.
- Richardson, P.M., McGuiness, U.M. and Aguayo, A.J. (1980). Axons from CNS neurons regenerate into PNS grafts. *Nature* **284**: 264-265.
- Richardson, P.M. and Ebendal, T. (1982). Nerve growth activities in the rat . *Brain Res* **246**: 57-64.
- Richardson, P.M., Issa, V. M.A. and Shemie, S. (1982a). Regeneration and retrograde degeneration of axons in the rat optic nerve. *J. Neurocytol* **11**: 949-966.
- Rosario, C. M., Aldskogius, H., Carlstedt, T. and Sidman, R.L. (1993). Differentiation and axonal outgrowth pattern of foetal dorsal root ganglion cells orthotopically allografted into adult rats. *Exp Neurol* **120**: 16-31
- Roskies, A. and O'Leary, D.D. (1994). Control of topographic retinal axon branching by inhibitory membrane-bound molecules. *Science* **265**: 799-803.
- Rudge, J.S. and Silver, J. (1990). Inhibition of neurite growth on astroglial scars *in vitro* *J. Neurosci* **10**: 3594-3603.
- Rustoni, A. and Weinberg, R.J. (1989). The somatosensory sensory system. In *handbook of chemical neuroanatomy. Vol 7 ; Integrated systems of the CNS*. Björklund, A., Hökfelt, T and Swanson, L.W. (eds). Elsevier. N.Y.
- Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S. (1992). Mice develop normally without tenascin. *Genes and Devel.* **6**: 1821-1831.

Sandrock, A.W. and Matthew, W.D. (1987). Identification of peripheral nerve neurite growth promoting activity by development and use of an *in vitro* bioassay. Proc Natl Acad Sci USA. **84**: 6934-6938.

Sanes, J.R. (1989). Extracellular matrix molecules that influence neural development. Ann Rev. Neurosci. **12**: 491-561.

Schnell, L. and Schwab, M.E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature **343**: 269-272.

Schnell, L., Schneider, R., Kolbeck, R., Barde, Y-A. and Schwab, M.E. (1994). Neurotrophin -3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature **367**: 170-173.

Schwab, M.E. and Thoenen, H. (1985). Dissociated neurons regenerate onto sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. J Neuroscience **5** 2415-2423.

Schwab, M.E. and Caroni, P. (1988). Oligodendrocytes and CNS myelin are non-permissive substrates for neurite growth and fibroblast spreading *in vitro*. J Neurosci **8**: 2381-2393.

Schwab, M.E. and Schnell, L. (1991). Channelling of developing rat corticospinal tract axons by myelin-associated neurite growth inhibitors. J. Neurosci **11**: 709-721.

Schwalb, J.M. Gu, M-f., Stuermer, C., Bastmeyer, M., Hu, G-f., Boulis, N., Irwin, N. and Benowitz, L.I. (1996). Optic nerve glia secrete a low molecular-weight factor that stimulates retinal ganglion cells to regenerate axons in goldfish. Neurosci **72**: 901-910.

Schwartz, J.P., Perarson, J. and Johnson, E.M. (1982). Effect of exposure to anti-NGF on sensory neurons adult rats and guinea pigs. Brain Res **244**: 378-381.

- Scott, S.A. (1992). Sensory Neurons. Diversity, Development and Plasticity. Oxford University Press. New York and Oxford.
- Seilheimer, B. and Schachner, M. (1988). Studies of adhesion molecules mediating interactions between cells of peripheral nervous system indicate a major role for L1 in mediating sensory neuron growth on Schwann cells in culture. *J Cell Biol* **107**: 341-351.
- Skene, J.H.P. and Willard, M. (1981). Characteristics of growth-associated proteins (GAPs) in regenerating toad retinal ganglion cells. *J Neurosci* **1**: 419-426.
- Smith, G.M., Rutishauser, U., Silver, J. and Miller, R.H. (1990). Maturation of astrocytes *in vitro* alters the extent and molecular basis of neurite outgrowth. *Dev Biol* **138**: 377-390.
- Smith-Thomas, L.C., Fok-Seang, J., Stevens, J., Du, J., Muir, E., Faissner, A., Geller, H M., Rogers, J. H. and Fawcett, J.W. (1994). An inhibitor of neurite outgrowth produced by astrocytes. *J Cell Sci* **107**: 1687-1695
- Snow, D.M., Lemmon, V., Carrino, D., Caplan, A. and Silver, J. (1990). Sulphated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. *Exp Neurol* **109**: 111-130.
- Son, Y-J. and Thompson, W.J. (1995). Schwann cell processes guide regeneration of peripheral axons. *Neuron* **14**: 125-132.
- Stöckli, K.A., Lottspeich, F., Sendter, M. and Masiakowski, P. (1989). Molecular cloning, Expression and regional distribution of rat ciliary neurotrophic factors. *Nature* **342**: 920-923.
- Taylor, J., Pesheva, P. and Schachner, M. (1993). the influence of Jenusin and Tenascin on the growth cone behaviour *in vitro*. *J Neurosci Res* **35**: 347-362.
- Tessler, A., Himes, B.T., Houle, J. and Reier, P.J. (1988). Regeneration of adult dorsal root axons into transplants of embryonic spinal cord. *J Comp Neurol* **270**: 537-548.

- Tiveron, M.C., Barboni, E., Pliego Rivero, F.B., Gormley, A.M., Seeley, P.J., Grosveld, F. and Morris, R. (1992). Selective inhibition of neurite outgrowth on mature astrocytes by Thy-1 glycoprotein. *Nature* **355**: 745-748
- Tiveron, M.C., Nosten-Bertrand, M., Jani, H., Garnett, D., Hirst, E.M.A., Grosveld, F. and Morris, R. (1994). The mode of anchorage to the cell surface determines both the function and the membrane location of Thy-1 glycoprotein. *J Cell Sci* **107**: 1783-1796.
- Thomas, K.R. and Capecchi, M.R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo derived stem cells. *Cell* **51**: 503-512.
- Tomaselli, K.J., Neugebauer, K.M., Bixby, J.L., Lilien, J. and Reichardt, L.F. (1988). N-cadherin and integrins: two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces. *Neuron* **1**: 33-43.
- Vidal-Sanz, M., Bray, G. M., Villegas-Perez, M.P., Thanos, S. and Aguayo, A. J. (1987). Axonal regeneration and synapse formation in the superior colliculus by retinal ganglion cells in the adult rat. *J Neurosci* **7**: 2984-2909.
- White, F.A., Silos-Santiago, I., Molliver, D.C., Nishimura, M., Phillips, H., Barbacid, M. and Snider, W.D. (1996). Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. *J Neurosci* **16**: 4662-4672.
- Whittemore, S.R., Larkfors, L., Ebendal, T., Holets, V.R., Ericsson, A. and Persson, H. (1987). Increased  $\beta$ -NGF messenger RNA and protein levels in neonatal rat hippocampus following specific cholinergic lesions. *J Neuroscience* **7**: 244-251.
- Victorin, K., Brundin, P., Gustavii, B., Lindvall, O. and Björklund, A. (1990). Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. *Nature* **347**: 556-558.
- Williams, A.F. (1989). The structure of Thy-1 antigen. pp. 49-70. *In* Cell surface antigen Thy-1 : Immunology, neurology and therapeutic applications. Ed : A.E. Reif and M. Schlessinger. Marcel Dekkar, Inc., New York and Basel.

Williams, E.J., Docherty, P., Turner, G., Reid, R.A. Hemperly, J.J. and Walsh, F.S. (1992). Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. *J Cell Biol* **119**: 883-892.

Willis, W.D. and Coggeshall, R.D. (1992). Sensory mechanisms of the spinal cord. pp 47-78. Academic press.

Winter, J., Forbes, A.C., Sternberg, J. and Lindsay, R.J. (1988). Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion (DRG) responses to the to the excitotoxin Capsaicin. *Neuron* **1**: 973-981.

Xue, G.P., Calvert, R. and Morris, R.J. (1990). Expression of the neuronal surface glycoprotein Thy-1 is under post-transcriptional control, and is spatially regulated in the developing olfactory system. *Development* **109**: 851-864.

Xue, G.P., Rivero, B.P. and Morris, R.J. (1991). The surface glycoprotein Thy-1 is excluded from growing axons during development : a study of the expression of Thy-1 during axonogenesis in hippocampus and hindbrain. *Development* **112**: 161-176.

Xue, G.P. and Morris, R.J. Expression of the neuronal surface glycoprotein Thy-1 does not follow appearance of its mRNA in developing mouse Purkinje cells. *J Neurochem* (1992). **58**: 430-440.

Xu, X.M., Guenard, V., Kleitman, N. and Bunge, M.B. (1994). Axonal regeneration in Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. *J Comp Neurol* **351**: 145-160.

Yasanobu, I. and Tessler, A. (1990). Regeneration of adult dorsal root axons into transplants of foetal spinal cord and brain: A comparison of growth and synapse formation in appropriate and inappropriate targets. *J Comp Neurol* **302**: 272-293.

Yasuda, T., Sobue, G., Ito, T., Mitsuma, T. and Takahashi, A. (1990). Nerve growth factors enhance neurite arborisation of adult sensory neurons : a study of single-cell culture. *Brain Res.* **534**: 54-63.

Yoshida, S. and Matsuda, Y. (1979). Studies on sensory neurons of the mouse with intracellular recording and dye injection techniques. *J Neurophysiol* **42**: 1134-1145.

Zhou, C. F., Raisman, G. and Morris, R. J. (1985). Specific pattern of fibre outgrowth from transplants to host mice hippocampi, shown immunohistochemically by the use of allelic forms of Thy-1.1. *Neurosci* **16**: 819-833.

Zhou, C.F., Li, Y. and Raisman, G. (1989). Embryonic entorhinal transplants project selectively to deafferented entorhinal zone of adult mouse hippocampi, as demonstrated by the use of Thy-1 allelic immunohistochemistry. Effect of timing of transplantation in relation to deafferentation. *Neurosci* **32**: 349-362.

Zhou, X. F, Williams, R., Zecevic, M. and Lush, R.A. (1994). Peripheral projections of a subpopulation of dorsal root ganglion neurons defined by ovalbumin immunoreactivity. *J of Neurocytol* **23**: 271-277.

Zwimpfer, T.J., Aguayo, A.J. and Bray, G.M. (1992). Synapse formation and preferential distribution in the granule cell layer by regenerating retinal ganglion cell axons guided to the cerebellum of adult hamsters. *J Neurosci* **12**: 1144-1159.